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**(54) Title:** FLK-1 IS A RECEPTOR FOR VASCULAR ENDOTHELIAL GROWTH FACTOR

FLK-1 866 ILIHGHLNVNLLGACTKPGCLMVIVFSKFGNLSTYLRGKRNEFVPYKSKGARFRQ  
 KDR \_\_\_\_\_ C — D — S — T —  
 TKR-C \_\_\_\_\_ C — — — — S

FLK-1 926 GKDYVGELSDVKRRLDSTISSQSSASSGFVEEKSLSQVEEEEASEELYKDFLTLEHLIC  
 KDR ——— AIP ————— P-D ———  
 TKR-C —————

FLK-1 986 YSFQVAKGMFLASRKCIHRDLAARNILLSEKNVVKICDFGLARDIYKDPDYVRKGDARL  
 KDR \_\_\_\_\_  
 TKR-C \_\_\_\_\_

**(57) Abstract**

The present invention relates to the use of ligands for the Flk-1 receptor for the modulation of angiogenesis and vasculogenesis. The invention is based, in part, on the demonstration that Flk-1 tyrosine kinase receptor expression is associated with endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. These results indicate a major role for Flk-1 in the signaling system during vasculogenesis and angiogenesis. Engineering of host cells that express Flk-1 and the uses of expressed Flk-1 to evaluate and screen for drugs and analogs of VEGF involved in Flk-1 modulation by either agonist or antagonist activities is described. The invention also relates to the use of FLK-1 ligands, including VEGF agonists and antagonists, in the treatment of disorders, including cancer, by modulating vasculogenesis and angiogenesis.

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-1-

Flk-1 IS A RECEPTOR FOR VASCULAR  
ENDOTHELIAL GROWTH FACTOR

1. INTRODUCTION

5       The present invention relates to the use of ligands  
for the FLK-1 receptor for the modulation of angiogenesis  
and vasculogenesis. The invention is based, in part, on  
the demonstration that Flk-1 tyrosine kinase receptor  
expression is associated with endothelial cells and the  
10       identification of vascular endothelial growth factor  
(VEGF) as the high affinity ligand of Flk-1. These  
results indicate a major role for Flk-1 in the signaling  
system during vasculogenesis and angiogenesis.  
Engineering of host cells that express Flk-1 and the uses  
15       of expressed Flk-1 to evaluate and screen for drugs and  
analogs of VEGF involved in Flk-1 modulation by either  
agonist or antagonist activities is described.

20       The invention also relates to the use of FLK-1  
ligands, including VEGF agonists and antagonists, in the  
treatment of disorders, including cancer, by modulating  
vasculogenesis and angiogenesis.

2. BACKGROUND OF THE INVENTION

25       Receptor tyrosine kinases comprise a large family of  
transmembrane receptors for polypeptide growth factors  
with diverse biological activities. Their intrinsic  
tyrosine kinase function is activated upon ligand  
binding, which results in phosphorylation of the receptor  
and multiple cellular substrates, and subsequently in a  
variety of cellular responses (Ullrich A. and  
30       Schlessinger, J., 1990, Cell 61:203-212).

35       A receptor tyrosine kinase cDNA, designated fetal  
liver kinase 1 (Flk-1), was cloned from mouse cell  
populations enriched for hematopoietic stem and  
progenitor cells. The receptor was suggested to be  
involved in hematopoietic stem cell renewal (Matthews

-2-

et al., 1991, Proc. Natl. Acad. Sci. USA 88:9026-9030). Sequence analysis of the Flk-1 clone revealed considerable homology with the c-Kit subfamily of receptor kinases and in particular to the Flt gene product. These receptors all have in common an extracellular domain containing immunoglobulin-like structures.

The formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus luteum during ovulation and placental growth after pregnancy. Uncontrolled angiogenesis can be pathological such as in the growth of solid tumors that rely on vascularization for growth.

Angiogenesis involves the proliferation, migration and infiltration of vascular endothelial cells, and is likely to be regulated by polypeptide growth factors. Several polypeptides with in vitro endothelial cell growth promoting activity have been identified. Examples include acidic and basic fibroblastic growth factor, vascular endothelial growth factor and placental growth factor. Although four distinct receptors for the different members of the FGF family have been characterized, none of these have as yet been reported to be expressed in blood vessels in vivo.

While the FGFs appear to be mitogens for a large number of different cell types, VEGF has recently been reported to be an endothelial cell specific mitogen (Ferrara, N. and Henzel, W.J., 1989, Biochem. Biophys. Res. Comm. 161:851-858). Recently, the fms-like tyrosine receptor, *flt*, was shown to have affinity for VEGF (DeVries, C. et al., 1992, Science 255:989-991).



-3-

### 3. SUMMARY OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor for the modulation of angiogenesis and vasculogenesis. The present invention is based, in part, on the discovery that the Flk-1 tyrosine kinase receptor is expressed on the surface of endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. The role of endothelial cell proliferation and migration during angiogenesis and vasculogenesis indicate an important role for Flk-1 in these processes. The invention is described by way of example for the murine Flk-1, however, the principles may be applied to other species including humans.

Pharmaceutical reagents designed to inhibit the Flk-1/VEGF interaction may be useful in inhibition of tumor growth. VEGF and/or VEGF agonists may be used to promote wound healing. The invention relates to expression systems designed to produce Flk-1 protein and/or cell lines which express the Flk-1 receptor. Expression of soluble recombinant Flk-1 protein may be used to screen peptide libraries for molecules that inhibit the Flk-1/VEGF interaction. Engineered cell lines expressing Flk-1 on their surface may be advantageously used to screen and identify VEGF agonists and antagonists.

### 4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Comparison of the Flk-1 amino acid sequence with related RTKs. Amino acid sequence comparison of Flk-1 with human KDR and rat TKr-C. A section of the sequence which is known for all three receptors is compared and only differences to the Flk-1 sequence are shown.

-4-

FIG. 2. Northern blot analysis of Flk-1 gene expression. (A) Expression of Flk-1 RNA in day 9.5 to day 18.5 mouse embryos. Samples (10  $\mu$ g) of total RNA from whole mouse embryos were analyzed in each lane. Positions of 28S and 18S ribosomal RNAs are marked. (B) Expression of Flk-1 mRNA in postnatal day 4 and adult brain in comparison with capillary fragments from postnatal day 4 brain. 1  $\mu$ g of poly (A<sup>+</sup>) RNA was loaded on each lane. The 5' 2619 bp of the Flk-1 cDNA were used as a probe. Control hybridization with a GAPDH cDNA probe is shown in the lower panel.

FIG. 3. Abundant Flk-1 gene expression in embryonic tissues. *In situ* hybridization analysis of Flk-1 expression in day 14.5 mouse embryo. (A) Bright field illumination of a parasagittal section through the whole embryo hybridized with a <sup>35</sup>S-labeled antisense probe (5' 2619 bp). (B) Dark field illumination of the same section. (C) Control hybridization of an adjacent section with a sense probe. Abbreviations: Ao, aorta; At, atrium; L, lung; Li, liver; Ma, mandible; Mn, meninges; Ms, mesencephalon; T, telencephalon; V, ventricle; Vt, vertebrae.

FIG. 4. Expression of Flk-1 RNA in embryonic organs is restricted to specific cells. Expression of Flk-1 RNA in a day 14.5 mouse embryo at higher magnification. (A) The heart region was probed with a <sup>35</sup>S-labeled antisense probe. (B) Adjacent section hybridized with the sense probe. (C) Part of the aorta wall shown on the cellular level. The endothelial cell layer is indicated by an arrow. (D) The lung, probed with the Flk-1 antisense probe. (E) Control hybridization of an adjacent section hybridized with the sense probe. Abbreviations: At, atrium; B, bronchus; Ed, endothelial cell layer; En, endocardium; L, lung, Li,

35

-5-

liver; Lu, lumina of the aorta; Ml, muscular; My, myocardium.

FIG. 5. Flk-1 gene expression in the brain of the developing mouse. In situ hybridization analysis of Flk-1 gene expression in the brain at different developmental stages. All sections were probed with the Flk-1 antisense probe. (A) Sagittal section of the telencephalon of a day 11.5 mouse embryo. A single blood vessel expressing Flk-1, which sprouts from the meninges into the neuroectoderm, is indicated by an arrow. (B) Sagittal sections of the brain of embryo day 14.5 and (C) of postnatal day 4. Shown are regions of the mesencephalon. Branching capillaries and blood vessels expressing Flk-1 are indicated by an arrow. (D) Sagittal section of an adult brain; a region of the mesencephalon is shown. Cells expressing Flk-1 are indicated by an arrow. Abbreviations: M, meninges; V, ventricle;

FIG. 6. Expression of Flk-1 in the choroid plexus of adult brain. (A) Darkfield illumination of the choroid plexus of an adult mouse brain hybridized with Flk-1 antisense probe. (B) Choroid plexus shown at a higher magnification. Arrows indicate single cells, which show strong expression of Flk-1. Abbreviations: CP, choroid plexus; E, ependyme; Ep, epithelial cells; V, ventricle.

FIG. 7. Flk-1 is expressed in the glomeruli of the kidney. (A) Parasagittal section of a 4-day postnatal kidney, hybridized with the Flk-1 antisense probe. Hybridization signal accumulates in the glomeruli, as indicated by arrowheads. (B) Control hybridization of an adjacent section with the sense probe. (C) Sagittal section of an adult kidney probed with Flk-1. Arrowheads indicate glomeruli. (D) Glomerulus of an adult kidney at a higher magnification. The arrows in (A) and (D)

-6-

indicate cells aligned in strands in the juxtaglomerular region expressing Flk-1.

FIG. 8. *In situ* hybridization analysis of Flk-1 expression in early embryos and extraembryonic tissues.

- 5 (A) Sagittal section of a day 8.5 mouse embryo in the maternal deciduum probed with Flk-1. (B) Higher magnification of the deciduum. Arrowheads indicate the endothelium of maternal blood vessels strongly expressing Flk-1 RNA. (C) High magnification of the yolk sac and  
10 the trophectoderm of a day 9.5 mouse embryo. (D) High magnification of a blood island. Abbreviations:  
A, allantois; Bi, blood island; Bv, maternal blood vessel; D, deciduum; En, endodermal layer of yolk sac; M, mesenchyme; Ms, mesodermal layer of yolk sac; NF,  
15 neural fold; T, trophoblast; Y, yolk sac.

- FIG. 9. Flk-1 is a receptor for VEGF. (A) Cross linking of  $^{125}\text{I}$ -VEGF to COS cells transiently expressing the Flk-1 receptor and control cells were incubated with  $^{125}\text{I}$ -VEGF at 4°C overnight, then washed twice with  
20 phosphate buffered saline (PBS) and exposed to 0.5 mM of the cross linking agent DSS in PBS for 1 hour at 4°C. The cells were lysed, Flk-1 receptor immunoprecipitated, and analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Molecular size markers are  
25 indicated in kilodaltons. (B) Specific binding of  $^{125}\text{I}$ -VEGF to COS cells expressing Flk-1. COS cells transiently expressing Flk-1 were removed from the plate and resuspended in binding medium (DMEM, 25 mM Hepes, 0.15% gelatin). Binding was performed at 15°C for 90  
30 minutes in a total volume of 0.5 ml containing  $2 \times 10^5$  cells, 15,000 cpm  $^{125}\text{I}$ -VEGF, and the indicated concentrations of unlabeled ligand. The cells were washed twice with PBS / 0.1% BSA and counted in a gamma  
35 counter.

-7-

FIG. 10. VEGF-induced autophosphorylation of Flk-1. COS cells transiently expressing Flk-1 receptor and control cells were starved for 24 hours in DMEM containing 0.5% fetal calf serum and then stimulated with VEGF for 10 minutes as indicated. The cells were solubilized, Flk-1 receptor immunoprecipitated with a polyclonal antibody against its C-terminus, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blot was probed with antiphosphotyrosine antibodies (5B2). The protein bands were visualized by using a horseradish-peroxidase coupled secondary antibody and BCL™ (Amersham) detection assay.

FIG. 11. Nucleotide Sequence of Murine Flk-1.

FIG. 12. Plasmid Maps of retroviral vector constructs. pLXSN Flk-1 TM Cl.1 and pLXSN Flk-1 TM cl.3 contain Flk-1 amino acids 1 through 806. pNTK-cfms-TM contains the 541 N-terminal amino acids of c-fms.

FIG. 13. Inhibition of C6 glioblastoma tumor growth by transdominant-negative inhibition of Flk-1. C6 cells were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cells lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-negative c-fms mutant (c-fms TM) as a control. Beginning at the time when the first tumors appeared, tumor volumes were measured every 2 to 3 days to obtain a growth curve. Each group is represented by four mice.

FIG. 14. Inhibition of C6 glioblastoma tumor growth by transdominant-negative inhibition of Flk-1. C6 cells were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cell lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-

-8-

negative c-fms mutant (cfms TM) as a control. Beginning at the time when the first tumor appeared, tumor volumes were measured every 2 to 3 days to obtain growth curve. Each group is represented by four mice.

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#### 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor to modulate angiogenesis and/or vasculogenesis. The invention also involves the  
10 expression of Flk-1 to evaluate and screen for drugs and analogs of VEGF that may be involved in receptor activation, regulation and uncoupling. Such regulators of Flk-1 may be used therapeutically. For example,  
agonists of VEGF may be used in processes such as wound  
15 healing; in contrast, antagonists of VEGF may be used in the treatment of tumors that rely on vascularization for growth.

The invention, is based, in part, on results from *in situ*-hybridization and Northern blot analyses indicating  
20 that Flk-1 is an endothelial cell specific RTK. In addition, cross-linking experiments have shown Flk-1 to be a high affinity receptor for vascular endothelial growth factor (VEGF), indicating that Flk-1 plays a crucial role in the development and differentiation of  
25 hemangioblast and in subsequent endothelial cell growth during vasculogenesis and angiogenesis.

The invention is based, also, on the discovery that expression of a transdominant-negative mutant form of the Flk-1 molecule can inhibit the biological activity of the  
30 endogenous wild type Flk-1. Experiments are described herein, in which tumor cells and cells producing a recombinant retrovirus encoding a truncated Flk-1 receptor were mixed and injected into mice. Inhibition of vasculogenesis and growth of the injected tumor cells  
35 was observed in mice expressing the truncated form of the

Flk-1 receptor. Expression of transdominant negative forms of the Flk-1 molecule may be useful for treatment of diseases resulting from abnormal proliferation of blood vessels, such as rheumatoid arthritis, retinopathies and growth of solid tumors.

As explained in the working examples, infra, the polymerase chain reaction (PCR) method was used to isolate new receptor tyrosine kinases specifically expressed in post-implantation embryos and endothelial cells. One such clone was found to encode a RTK that had almost identical sequence homology with the previously identified cDNA clone isolated from populations of cells enriched for hematopoietic cells and designated fetal liver kinase-1 (Flk-1) (Matthews et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030) (FIG. 11).

For clarity of discussion, the invention is described in the subsections below by way of example for the murine Flk-1. However, the principles may be analogously applied to clone and express the Flk-1 of other species including humans.

#### 5.1. THE Flk-1 CODING SEQUENCE

The nucleotide coding sequence and deduced amino acid sequence of the murine Flk-1 gene is depicted in Figure 11 (SEQ. ID NO. 1) and has recently been described in Matthews et al., 1991, Proc. Natl. Acad. Sci. U.S.A., 88:9026-9030. In accordance with the invention, the nucleotide sequence of the Flk-1 protein or its functional equivalent in mammals, including humans, can be used to generate recombinant molecules which direct the expression of Flk-1; hereinafter, this receptor will be referred to as "Flk-1", regardless of the species from which it is derived.

In a specific embodiment described herein, the murine Flk-1 gene was isolated by performing a polymerase

-10-

chain reaction (PCR) using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of receptor tyrosine kinases (Hanks et al., 1988,) As a template,  
5 DNA from a  $\lambda$ gt10 cDNA library prepared from day 8.5 mouse embryos, was used. In a parallel approach, similar primers were used to amplify RTK cDNA sequences from capillary endothelial cells that had been isolated from the brains of post-natal day 4-8 mice. This is a time  
10 when brain endothelial cell proliferation is maximal. Both approaches yielded cDNA sequences encoding the recently described fetal liver RTK, Flk-1 (Matthews et al., 1991). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich and  
15 Schlessinger) which contain immunoglobulin-like repeats in their extracellular domains (FIG. 1).

The invention also relates to Flk-1 genes isolated from other species, including humans, in which Flk-1 activity exists. Members of the Flk-1 family are defined  
20 herein as those receptors that bind VEGF or fragments of the peptide. Such receptors may demonstrate about 80% homology at the amino acid level in substantial stretches of DNA sequence. A bacteriophage cDNA library may be screened, under conditions of reduced stringency, using a  
25 radioactively labeled fragment of the mouse Flk-1 clone. Alternatively the mouse Flk-1 sequence can be used to design degenerate or fully degenerate oligonucleotide probes which can be used as PCR probes or to screen bacteriophage cDNA libraries. A polymerase chain  
30 reaction (PCR) based strategy may be used to clone human Flk-1. Two pools of degenerate oligonucleotides, corresponding to a conserved motifs between the mouse Flk-1 and receptor tyrosine kinases, may be designed to serve as primers in a PCR reaction. The template for the  
35 reaction is cDNA obtained by reverse transcription of



-11-

mRNA prepared from cell lines or tissue known to express human Flk-1. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the Flk-1 sequences. The PCR fragment may be used to isolate a full length Flk-1 cDNA clone by radioactively labeling the amplified fragment and screening a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used, see e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.)

Isolation of a human Flk-1 cDNA may also be achieved by construction of a cDNA library in a mammalian expression vector such as pcDNA1, that contains SV40 origin of replication sequences which permit high copy number expression of plasmids when transferred into COS cells. The expression of Flk-1 on the surface of transfected COS cells may be detected in a number of ways, including the use of a labeled ligand such as VEGF or a VEGF agonist labeled with a radiolabel, fluorescent label or an enzyme. Cells expressing the human Flk-1 may be enriched by subjecting transfected cells to a FACS (fluorescent activated cell sorter) sort.

In accordance with the invention, Flk-1 nucleotide sequences which encode Flk-1, peptide fragments of Flk-1, Flk-1 fusion proteins or functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of Flk-1 protein or a functionally equivalent thereof, in appropriate host cells. Alternatively, nucleotide sequences which hybridize to portions of the Flk-1 sequence may also be used in

-12-

nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the Flk-1 protein. Such DNA sequences include those which are capable of hybridizing to the murine Flk-1 sequence under stringent conditions.

10 Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may

15 contain deletions, additions or substitutions of amino acid residues within the Flk-1 sequence, which result in a silent change thus producing a functionally equivalent Flk-1. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility,

20 hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups

25 having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. As used herein, a functionally equivalent Flk-1 refers to a receptor which binds to VEGF or fragments,

30 but not necessarily with the same binding affinity of its counterpart native Flk-1.

The DNA sequences of the invention may be engineered in order to alter the Flk-1 coding sequence for a variety of ends including but not limited to alterations which

35 modify processing and expression of the gene product.

-13-

For example, mutations may be introduced using techniques which are well known in the art, e.g. site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc. For  
5 example, in certain expression systems such as yeast, host cells may over glycosylate the gene product. When using such expression systems it may be preferable to alter the Flk-1 coding sequence to eliminate any N-linked glycosylation site.

10 In another embodiment of the invention, the Flk-1 or a modified Flk-1 sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries it may be useful to encode a chimeric Flk-1 protein expressing a  
15 heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the Flk-1 sequence and the heterologous protein sequence, so that the Flk-1 can be cleaved away from the heterologous  
20 moiety.

In an alternate embodiment of the invention, the coding sequence of Flk-1 could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, Nuc. Acids  
25 Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers, 1980, Tetrahedron Letters 21:719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods  
30 to synthesize the Flk-1 amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (E.g., see Craighton, 1983, Proteins  
35 Structures And Molecular Principles, W.H. Freeman and

-14-

Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49.

## 5.2. EXPRESSION OF Flk-1 RECEPTOR AND GENERATION OF CELL LINES THAT EXPRESS Flk-1

In order to express a biologically active Flk-1, the nucleotide sequence coding for Flk-1, or a functional equivalent as described in Section 5.1 supra, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The Flk-1 gene products as well as host cells or cell lines transfected or transformed with recombinant Flk-1 expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (i.e., monoclonal or polyclonal) that bind to the receptor, including those that competitively inhibit binding of VEGF and "neutralize" activity of Flk-1 and the screening and selection of VEGF analogs or drugs that act via the Flk-1 receptor; etc.

### 25 5.2.1. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the Flk-1 coding sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular

-15-

Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the Flk-1 coding sequence. These  
5 include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the Flk-1 coding sequence; yeast transformed with  
10 recombinant yeast expression vectors containing the Flk-1 coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the Flk-1 coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus;  
15 TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the Flk-1 coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) including cell lines engineered to  
20 contain multiple copies of the Flk-1 DNA either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines).

The expression elements of these systems vary in their strength and specificities. Depending on the  
25 host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of  
30 bacteriophage  $\lambda$ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant  
35 cells (e.g., heat shock promoters; the promoter for the

-16-

small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the Flk-1 DNA SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the Flk-1 expressed. For example, when large quantities of Flk-1 are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the Flk-1 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

-17-

- In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.
- 15 In cases where plant expression vectors are used, the expression of the Flk-1 coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

-18-

An alternative expression system which could be used to express Flk-1 is an insect system. In one such system, Autographa californica nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes.

5 The virus grows in Spodoptera frugiperda cells. The Flk-1 coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion  
10 of the Flk-1 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera  
15 frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an  
20 adenovirus is used as an expression vector, the Flk-1 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in  
25 vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing Flk-1 in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA)  
30 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

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-19-

Specific initiation signals may also be required for efficient translation of inserted Flk-1 coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire Flk-1 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the Flk-1 coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the Flk-1 coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such

-20-

mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, 5 cell lines which stably express the Flk-1 may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the Flk-1 DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, 10 sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable 15 marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell 20 lines which express the Flk-1 on the cell surface, and which respond to VEGF mediated signal transduction. Such engineered cell lines are particularly useful in screening VEGF analogs.

A number of selection systems may be used, including 25 but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 30 22:817) genes can be employed in tk<sup>-</sup>, hgp<sup>r</sup>t<sup>-</sup> or ap<sup>r</sup>t<sup>-</sup> cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. 35 Acad. Sci. USA 78:1527); gpt, which confers resistance to

-21-

mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygromycin (Santerre, et al., 1984, Gene 30:147) to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

#### 5.2.2. IDENTIFICATION OF TRANSFECTANTS OR TRANSFORMANTS THAT EXPRESS THE Flk-1

The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of Flk-1 mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the Flk-1 coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the Flk-1 coding sequence, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based

-22-

upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the Flk-1 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the Flk-1 coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the Flk-1 sequence under the control of the same or different promoter used to control the expression of the Flk-1 coding sequence. Expression of the marker in response to induction or selection indicates expression of the Flk-1 coding sequence.

In the third approach, transcriptional activity for the Flk-1 coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the Flk-1 coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the Flk-1 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active Flk-1 gene product. A number of assays can be used to detect receptor activity including but not limited to VEGF binding assays; and VEGF biological assays using engineered cell lines as the test substrate.

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-23-

### 5.3. USES OF THE Flk-1 RECEPTOR AND ENGINEERED CELL LINES

Angiogenesis, the growth of new blood capillary vessels, is required for a number of physiological processes ranging from wound healing, tissue and organ regeneration, placental formation after pregnancy and embryonic development. Abnormal proliferation of blood vessels is an important component of a variety of diseases such as rheumatoid arthritis, retinopathies, and psoriasis. Angiogenesis is also an important factor in the growth and metastatic activity of solid tumors that rely on vascularization. Therefore, inhibitors of angiogenesis may be used therapeutically for the treatment of diseases resulting from or accompanied by abnormal growth of blood vessels and for treatments of malignancies involving growth and spread of solid tumors.

In an embodiment of the invention the Flk-1 receptor and/or cell lines that express the Flk-1 receptor may be used to screen for antibodies, peptides, or other ligands that act as agonists or antagonists of angiogenesis or vasculogenesis mediated by the Flk-1 receptor. For example, anti-Flk-1 antibodies capable of neutralizing the activity of VEGF, may be used to inhibit Flk-1 function. Additionally, anti-Flk-1 antibodies which mimic VEGF activity may be selected for uses in wound healing. Alternatively, screening of peptide libraries with recombinantly expressed soluble Flk-1 protein or cell lines expressing Flk-1 protein may be useful for identification of therapeutic molecules that function by inhibiting the biological activity of Flk-1.

In an embodiment of the invention, engineered cell lines which express the entire Flk-1 coding region or its ligand binding domain may be utilized to screen and identify VEGF antagonists as well as agonists. Synthetic compounds, natural products, and other sources of

potentially biologically active materials can be screened in a number of ways. The ability of a test compound to inhibit binding of VEGF to Flk-1 may be measured using standard receptor binding techniques, such as those described in Section 6.1.9. The ability of agents to prevent or mimic, the effect of VEGF binding on signal transduction responses on Flk-1 expressing cells may be measured. For example, responses such as activation of Flk-1 kinase activity, modulation of second messenger production or changes in cellular metabolism may be monitored. These assays may be performed using conventional techniques developed for these purposes.

#### 5.3.1. SCREENING OF PEPTIDE LIBRARY WITH Flk-1 PROTEIN OR ENGINEERED CELL LINES

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Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to the ligand binding site of a given receptor or other functional domains of a receptor such as kinase domains (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to inhibit the biological activity of receptors through their interactions with the given receptor.

Identification of molecules that are able to bind to the Flk-1 may be accomplished by screening a peptide library with recombinant soluble Flk-1 protein. Methods for expression and purification of Flk-1 are described in Section 5.2.1 and may be used to express recombinant full length Flk-1 or fragments of Flk-1 depending on the functional domains of interest. For example, the kinase and extracellular ligand binding domains of Flk-1 may be separately expressed and used to screen peptide libraries.

-25-

To identify and isolate the peptide/solid phase support that interacts and forms a complex with Flk-1, it is necessary to label or "tag" the Flk-1 molecule. The Flk-1 protein may be conjugated to enzymes such as  
5 alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label, to Flk-1, may be performed using techniques that are routine  
10 in the art. Alternatively, Flk-1 expression vectors may be engineered to express a chimeric Flk-1 protein containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including  
15 labeling with enzymes, fluorescent dyes or colored or magnetic beads.

The "tagged" Flk-1 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between Flk-1 and peptide  
20 species within the library. The library is then washed to remove any unbound Flk-1 protein. If Flk-1 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing a substrates for either alkaline phosphatase  
25 or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diaminobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-Flk-1 complex changes color, and can be easily identified and isolated physically under a  
30 dissecting microscope with a micromanipulator. If a fluorescent tagged Flk-1 molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric Flk-1 protein expressing a heterologous epitope has been used, detection of the  
35 peptide/Flk-1 complex may be accomplished by using a

-26-

labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

In addition to using soluble Flk-1 molecules, in another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with receptors that require the lipid domain of the cell membrane to be functional. Methods for generating cell lines expressing Flk-1 are described in Sections 5.2.1. and 5.2.2. The cells used in this technique may be either live or fixed cells. The cells will be incubated with the random peptide library and will bind to certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes where label or "tag" can be attached.

### 5.3.2. ANTIBODY PRODUCTION AND SCREENING

Various procedures known in the art may be used for the production of antibodies to epitopes of the recombinantly produced Flk-1 receptor. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Neutralizing antibodies i.e., those which compete for the VEGF binding



-27-

site of the receptor are especially preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind Flk-1 may be radioactively labeled allowing one to follow their location and distribution in the body after injection. Radioactivity tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* vascularization associated with a number of diseases including rheumatoid arthritis, macular degeneration, and formation of tumors and metastases.

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity Flk-1 specific monoclonal antibodies may be covalently complexed to bacterial or plant toxins, such as diphtheria toxin, abrin or ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin to the antibody. The hybrid antibodies may be used to specifically eliminate Flk-1 expressing endothelial cells.

For the production of antibodies, various host animals may be immunized by injection with the Flk-1 protein including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

-28-

Monoclonal antibodies to Flk-1 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce Flk-1-specific single chain antibodies.

Antibody fragments which contain specific binding sites of Flk-1 may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to Flk-1.

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5.4. USES OF Flk-1 CODING SEQUENCE

-29-

The Flk-1 coding sequence may be used for diagnostic purposes for detection of Flk-1 expression. Included in the scope of the invention are oligoribonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes that function to inhibit translation of Flk-1. In addition, mutated forms of Flk-1, having a dominant negative effect, may be expressed in targeted cell populations to inhibit the activity of endogenously expressed wild-type Flk-1.

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#### 5.4.1. USE OF Flk-1 CODING SEQUENCE IN DIAGNOSTICS AND THERAPEUTICS

The Flk-1 DNA may have a number of uses for the diagnosis of diseases resulting from aberrant expression of Flk-1. For example, the Flk-1 DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of Flk-1 expression; e.g., Southern or Northern analysis, including in situ hybridization assays.

20 The Flk-1 cDNA may be used as a probe to detect the expression of the Flk-1 mRNA. In a specific example described herein, the expression of Flk-1 mRNA in mouse embryos of different developmental stages was analyzed. Northern blot analysis indicated abundant expression of a major 5.5 kb mRNA between day 9.5 and day 18.5, with apparent decline towards the end of gestation (FIG. 2A). In post-natal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain RNA (FIG.2B), suggesting a role for Flk-1 in endothelial cell proliferation.

30 To obtain more detailed information about the expression of Flk-1 during embryonic development and during the early stages of vascular development in situ hybridization experiments were performed as described in Section 6.1.4. In situ hybridizations demonstrated that

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-30-

Flk-1 expression in vivo during embryonic mouse development is largely restricted to endothelial cells and their precursors (FIG. 3 and FIG. 4). Flk-1 is expressed in endothelial cells during physiological processes that are characterized by endothelial cell proliferation and the temporal and spatial expression pattern found in the embryonic brain correlate precisely with the development of the neural vascular system as described by Bar (1980). Vascular sprouts originating in the perineural plexus grow radially into the neuroectoderm and branch there and these sprouts were found to express high amounts of Flk-1 mRNA (FIG. 5). In the early postnatal stages endothelial cell proliferation is still evident and Flk-1 is expressed, whereas in the adult organism, after completion of the vascularization process, the decline in endothelial cell proliferation parallels a decrease in Flk-1 expression.

Also within the scope of the invention are oligoribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of Flk-1 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the Flk-1 nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and

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-31-

efficiently catalyze endonucleolytic cleavage of Flk-1 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends

-32-

of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

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5.4.2. USE OF DOMINANT NEGATIVE  
Flk-1 MUTANTS IN GENE THERAPY

Receptor dimerization induced by ligands, is thought to provide an allosteric regulatory signal that functions to couple ligand binding to stimulation of kinase  
10 activity. Defective receptors can function as dominant negative mutations by suppressing the activation and response of normal receptors by formation of unproductive heterodimers. Therefore, defective receptors can be  
15 engineered into recombinant viral vectors and used in gene therapy in individuals that inappropriately express Flk-1.

In an embodiment of the invention, mutant forms of the Flk-1 molecule having a dominant negative effect may be identified by expression in selected cells. Deletion  
20 or missense mutants of Flk-1 that retain the ability to form dimers with wild type Flk-1 protein but cannot function in signal transduction may be used to inhibit the biological activity of the endogenous wild type Flk-1. For example, the cytoplasmic kinase domain of Flk-1  
25 may be deleted resulting in a truncated Flk-1 molecule that is still able to undergo dimerization with endogenous wild type receptors but unable to transduce a signal.

Abnormal proliferation of blood vessels is an  
30 important component of a variety of pathogenic disorders such as rheumatoid arthritis, retinopathies and psoriasis. Uncontrolled angiogenesis is also an important factor in the growth and metastases of solid tumors. Recombinant viruses may be engineered to express  
35 dominant negative forms of Flk-1 which may be used to

-33-

inhibit the activity of the wild type endogenous Flk-1. These viruses may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of Flk-1.

5        Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant Flk-1 into the targeted cell population. Methods which are well known to those  
10 skilled in the art can be used to construct recombinant viral vectors containing Flk-1 coding sequence. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current  
15 Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant Flk-1 molecules can be reconstituted into liposomes for delivery to target cells.

      In a specific embodiment of the invention, a  
20 deletion mutant of the Flk-1 receptor was engineered into a recombinant retroviral vector. Two clonal isolates designated pLXSN Flk-1 TM cl.1 and pLXSN Flk-1 TM cl.3 contain a truncated Flk-1 receptor lacking the 561 COOH-terminal amino acids. To obtain virus producing cell  
25 lines, PA37 cells were transfected with the recombinant vectors and, subsequently, conditioned media containing virus were used to infect GPE cells.

      To test whether expression of signaling-defective mutants inhibits endogenous Flk-1 receptor activity, C6  
30 rat glioblastoma cells (tumor cells) and mouse cells producing the recombinant retroviruses were mixed and injected subcutaneously into nude mice. Normally, injection of tumor cells into nude mice would result in proliferation of the tumor cells and vascularization of  
35 the resulting tumor mass. Since Flk-1 is believed to be

-34-

essential for formation of blood vessels, blocking Flk-1 activity by expression of a truncated receptor, might function to inhibit vascularization of the developing tumor and, thereby, inhibit its growth. As illustrated in Figures 13 and 14, coinjection of virus producing cells, expressing a truncated Flk-1 receptor, significantly inhibits the growth of the tumor as compared to controls receiving only tumor cells.

#### 5.5. USE OF Flk-1 RECEPTOR OR LIGANDS

Receptor/ligand interaction between Flk-1 and VEGF is believed to play an important role in the signalling system during vascularization and angiogenesis. Abnormal proliferation of blood vessels is an important component of a number of diseases.

Expression of Flk-1 RNA correlates with the development of the brain and with endothelial cell proliferation suggesting that Flk-1 might be a receptor involved in mediation of signaling events in the vascularization process. VEGF has been shown to be a mitogenic growth factor known to act exclusively on endothelial cell (Ferrara, N. and Henzel, W.J., 1989, Biochem. Biophys. Res. Comm. 161:851-858). Cross-linking and ligand binding experiments were performed, as described in Section 6.1.9 and 6.1.10 respectively, to determine whether VEGF is a ligand for Flk-1 and the results indicate that Flk-1 is an authentic high affinity VEGF receptor (FIG 9).

In one embodiment of the invention, ligands for Flk-1, the Flk-1 receptor itself, or a fragment containing its VEGF binding site, could be administered in vivo to modulate angiogenesis and/or vasculogenesis. For example, administration of the Flk-1 receptor or a fragment containing the VEGF binding site, could competitively bind to VEGF and inhibit its interaction



-35-

with the native Flk-1 receptor in vivo to inhibit angiogenesis and/or vasculogenesis. Alternatively, ligands for Flk-1, including anti-Flk-1 antibodies or fragments thereof, may be used to modulate angiogenesis and/or vasculogenesis. Agonists of VEGF activity may be used to promote wound healing whereas antagonists of VEGF activity may be used to inhibit tumor growth.

Depending on the specific conditions being treated, these agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. Suitable routes may include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

6. EXAMPLE: CLONING AND EXPRESSION PATTERNS  
OF Flk-1, A HIGH AFFINITY  
RECEPTOR FOR VEGF

The subsection below describes the cloning and characterization of the Flk-1 cDNA clone. Northern blot and *in situ* hybridization analyses indicate that Flk-1 is expressed in endothelial cells. Cross-linking and ligand binding experiments further indicate that Flk-1 is a high affinity receptor for VEGF.

## 6.1. MATERIALS AND METHODS

### 6.1.1. CDNA CLONING OF Flk-1

DNA extracted from  $\lambda$ gt10 cDNA library of day 8.5  
5 mouse embryos (Fahrner et al., 1987, EMBO. J. 6:1497-1508) was used as template for polymerase chain reaction (PCR; Saiki, R.K. et al., 1985 Science 230:1350-1354). In an independent approach cDNA of capillary endothelial cells that had been isolated from the brain of postnatal  
10 day 4-8 mice was used for amplification (Risau, W., 1990 In: development of the Vascular System. Issues Biomed. Basel Karger 58-68 and Schnürch et al., unpublished) Degenerated primers were designed on the basis of high amino acid homologies within the kinase domain shared by  
15 all RTKs (Wilks, A.F., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:1603-1607).

Full length cDNA clones of Flk-1 were isolated from another day 8.5 mouse embryo cDNA library, which had been prepared according to the method of Okayama and Berg  
20 (1983), and a day 11.5 mouse embryo  $\lambda$ gt11 library (Clontech) using the  $^{32}$ P-labeled (Feinberg, A.P. and Vogelstein, B. 1983 Anal. Biochem. 132:6-13) 210-bp PCR fragment.

### 25 6.1.2. MOUSE EMBRYOS

Balb/c mice were mated overnight and the morning of vaginal plug detection was defined as 1/2 day of gestation. For Northern blot analysis the frozen embryos were homogenized in 5 M guanidinium thiocyanate and RNA  
30 was isolated as described (Ullrich, A. et al., 1985, Nature 313:756-761). For in situ hybridization, the embryos were embedded in Tissue-Tek (Miles), frozen on the surface of liquid nitrogen and stored at -70C prior to use.

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-37-

### 6.1.3. PREPARATION OF PROBES

The 5'-located 2619 bp of the receptor cDNA were subcloned in the pGem3Z vector (Promega) as an EcoRI/BamHI fragment. The probe for Northern blot hybridization was prepared by labelling the cDNA fragment with  $\alpha$ -<sup>32</sup>PdATP (Amersham) by random hexanucleotide priming (Boehringer; Feinberg, A.P. and Vogelstein, B., 1983 Anal. Biochem. 132:6-13).

For in situ hybridization a single-strand antisense DNA probe was prepared as described by Schnürch and Risau (Development, 1991 111:1143-54). The plasmid was linearized at the 3' end of the cDNA and a sense transcript was synthesized using SP6 RNA polymerase (Boehringer). The DNA was degraded using DNAase (RNAase free preparation, Boehringer Mannheim). With the transcript, a random-primed cDNA synthesis with a  $\alpha$ -<sup>35</sup>S dATP (Amersham) was performed by reverse transcription with MMLV reverse transcriptase (BRL). To obtain small cDNA fragments of about 100 bp in average suitable for in situ hybridization, a high excess of primer was used. Subsequently the RNA transcript was partially hydrolyzed in 100 mM NaOH for 20 minutes at 70°C, and the probe was neutralized with the same amount of HCl and purified with a Sephadex C50 column. After ethanol precipitation the probe was dissolved at a final specific activity of  $5 \times 10^5$  cpm. For control hybridization a sense probe was prepared with the same method.

### 6.1.4. RNA EXTRACTION AND NORTHERN ANALYSIS

Total cytoplasmic RNA was isolated according to the acidic phenol-method of Chromczynski and Sacchi (1987). Poly(A<sup>+</sup>) RNA aliquots were electrophoresed in 1.2% agarose formaldehyde (Sambrook, J. et al., 1989 Molecular Cloning: A Laboratory Manual 2nd ed. Cold Spring Harbor Laboratory Press) gels and transferred to nitrocellulose

-38-

membranes (Schleicher & Schuell), Hybridizations were performed overnight in 50% formamide, 5 x SSC (750mM sodium chloride, 75mM sodium citrate), 5 x Denhardt's (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA) and -0.5% SDS at 42°C with  $1-3 \times 10^6$  cpm-ml<sup>-1</sup> of <sup>32</sup>P-Random primed DNA probe, followed by high stringency washes in 0.2 x SSC, 0.5% SDS at 52°C. The filters were exposed for 4 to 8 days.

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#### 6.1.5. IN SITU HYBRIDIZATION

Subcloning postfixation and hybridization was essentially performed according to Hogan et al. (1986). 10 µm thick sections were cut at -18°C on a Leitz cryostat. For prehybridization treatment no incubation with 0.2M HCl for removing the basic proteins was performed. Sections were incubated with the <sup>35</sup>S-cDNA probe ( $5 \times 10^4$  cpm/µl) at 52°C in a buffer containing 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, 10 mM NaPO<sub>4</sub> (pH 6.8), 5 mM EDTA, 0.02% Ficoll 400, 0.01% polyvinylpyrrolidone 0.02% BSA 10 mg/ml yeast RNA, 10% dextran sulfate, and 10 mM NaCl, 10 mM Tris-HCl, 10 mM NaPO<sub>4</sub> (pH 6.8), 5 mM EDTA, 10 mM DTT at 52°C). For autoradiography, slides were coated with Kodak NTB2 film emulsion and exposed for eight days. After developing, the sections were counterstained and toluidine blue or May-Grünwald.

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#### 6.1.6. PREPARATION OF ANTISERA

The 3' primed EcoRV/HindII fragment comprising the 128 C-terminal amino acids of Flk-1 was subcloned in the fusion protein expression vector pGEX3X (Smith, D.B. and Johnson, K.S., 1990 Gene. 67:31-40; Pharmacia). The fusion protein was purified as described and used for immunizing rabbits. After the second boost the rabbits

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-39-

were bled and the antiserum was used for immunoprecipitation.

6.1.7. TRANSIENT EXPRESSION  
OF Flk-1 IN COS-1 CELLS

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Transfection of COS-1 cells was performed essentially as described by Chen and Okayama (1987 Mol. Cell. Biol. 7:2745-2752) and Gorman et al. (1989 Virology 171:377-385). Briefly, cells were seeded to a density of  
10  $1.0 \times 10^6$  per 10-cm dish and incubated overnight in DMEM containing 10% fetal calf serum (Gibco). 20  $\mu$ g of receptor cDNA cloned into a cytomegalovirus promotor driven expression vector was mixed in 0.5 ml of 0.25 M  $\text{CaCl}_2$ , 0.5 ml of 2 x BBS (280 mM NaCl, 1.5 mM  $\text{Na}_2\text{HPO}_4$ , 50  
15 mM BES, pH 6.96 and incubated for 30 min at room temperature. The calcium phosphate/DNA solution was then added to the cells, swirled gently, and incubated for 18 hours at 37°C under 3%  $\text{CO}_2$ . For ligand binding experiments, the cells were removed from the plate and  
20 treated as described below.

To obtain VEGF conditioned media, cells were transfected in 15-cm dishes. Media was collected after 48 h and VEGF was partially purified by affinity chromatography using heparin High Trap <sup>TM</sup> columns  
25 (Pharmacia) and concentrated by ultrafiltration (Ferrara, N. and Henzel, W.J. 1989 Biochem. Biophys. Res. Comm. 161:851-858). The concentration of VEGF was determined by a ligand competition assay with bovine aortic endothelial cells.

30 For autophosphorylation assays, cells were seeded in 6-well dishes ( $2 \times 10^5$  cells per well), transfected as described above, and starved for 24 h in DMEM containing 0.5% fetal calf serum. The cells were then treated with 500 pM VEGF for 10 min. at 37°C or left untreated and  
35 were subsequently lysed as described by Kris et al.

-40-

(1985). Flk-1 was immunoprecipitated with an antiserum raised in rabbits against the C-terminus of the receptor. The immunoprecipitates were separated on a 7.5% SDS polyacrylamide gel, transferred to nitrocellulose, and  
5 incubated with a mouse monoclonal antibody directed against phosphotyrosine (5E2; Fendly, B.M. et al., 1990 Cancer Research 50:1550-1558). Protein bands were visualized using horseradish peroxidase coupled goat anti-mouse antibody and the ECL<sup>TM</sup> (Amersham) detection  
10 system.

#### 6.1.8. RADIOIODINATION OF VEGF

Recombinant human VEGF (5 µg; generously provided by Dr. H. Weich) was dissolved in 110 µl sodium phosphate  
15 buffer pH 7.6, and iodinated by the procedure of Hunter and Greenwood (1962). The reaction products were separated from the labeled protein by passage over a sephadex G50 column, pre-equilibrated with phosphate buffered saline (PBS) containing 0.7% bovine serum  
20 albumin (BSA), and aliquots of the collected fractions were counted before and after precipitation with 20% trichloroacetic acid. The purity of the iodinated product was estimated to be superior to 90%, as determined by gel electrophoresis, and the specific activity was 77000  
25 cpm/ng. The bioactivity of the iodinated VEGF was confirmed by comparison with the bioactivities of native VEGF using the tissue factor introduction assay described by Clauss, M. et al. (1990 J. Exp. Med. 172:1535-1545).

#### 30 6.1.9. CROSSLINKING OF VEGF TO Flk-1

COS-1 cells transiently expressing Flk-1 and untransfected COS-1 cells were incubated with 200 pM <sup>125</sup>I-VEGF at 4°C overnight, then washed twice with PBS and exposed to 0.5 mM disuccinimidyl suberate (DSS) in PBS  
35 for 1 h at 4°C. The cells were lysed, Flk-1

-41-

immunoprecipitated, and analyzed by electrophoresis on a 7% polytarcylamide gel followed by autoradiography.

#### 6.1.10. VEGF BINDING

5       Ligand binding experiments were performed as described previously (Schumacher, R. et al., 1991, J. Biol. Chem. 266:19288-19295), COS-1 cells were grown in a 15-cm culture dish in DMEM for 48h after transfection. Cells were then washed carefully with PBS and incubated  
10   with 5 ml of 25 mM EDTA in PBS for 10 min. Cells were then removed from the plate, washed once with binding buffer (DMEM, 25 mM HEPES, pH 7.5, 0.15% gelatin) and resuspended in 5 ml of binding buffer to determine the cell number. In a total volume of 500  $\mu$ l this cell  
15   suspension was incubated for 90 min at 15°C with 10 pM  $^{125}$ I-VEGF, and increasing concentration of unlabeled ligand (from 0 to  $7 \times 10^{-9}$ ), which was partially purified from conditioned media of COS-1 cells transiently expressing VEGF (164 amino acid form; Breier et al., 1992). After  
20   incubation, cells were washed with PBS 0.1% PBS in the cold. Free ligand was removed by repeated centrifugation and resuspension in binding buffer. Finally, the  $^{125}$ I radioactivity bound to the cells were determined in a gamma counter (Riastar). Data obtained were analyzed by  
25   the method of Munson, P.J. and Rodbard, D. (1980 Anal. Biochem. 107:220-235).

#### 6.1.11. RETROVIRAL VECTORS ENCODING TRANSDOMINANT-NEGATIVE MUTANTS OF Flk-1

30       Recombinant retroviral vectors were constructed that contained the coding region for amino acids 1 through 806 of the Flk-1 receptor (pLX Flk-1 cl.1 and cl.3, Figure 12). A recombinant virus containing a truncated c-fms  
35   receptor mutant (pNTK cfms TM cl.7) was used as a control. To obtain virus producing cells mouse GPE cells

-42-

were infected with amphotrophic virus-containing conditioned media of PA317 cells that had been transfected with recombinant retroviral DNA. C6 glioblastoma tumor cells were implanted into nude mice either alone or coimplanted with virus producing cells. Injected cell numbers for the two sets of experiments are indicated below. Beginning at the time when the first tumors appeared, tumor volumes were measured every 2 to 3 days to obtain a growth curve.

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## Experiment No. 1

Number of Mice	Number of C6 Cells	Virus-Producer Cell Line	Number of Virus-Cells
4	$5 \times 10^5$	pLXSN Flk-1 TM cl.3	$1 \times 10^7$
4	$5 \times 10^5$	None	0
4	$5 \times 10^5$	pNTK cfms TM cl.7	$5 \times 10^6$

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## Experiment No. 2

Number of Mice	Number of C6 Cells	Virus-Producer Cell Line	Number of Virus-Cells
4	$2 \times 10^6$	pLXSN Flk-1 TM cl.1	$2 \times 10^7$
4	$2 \times 10^6$	pLXSN Flk-1 TM cl.3	$2 \times 10^7$
4	$2 \times 10^6$	None	0
4	$2 \times 10^6$	pNTK cfms TM cl.7	$2 \times 10^7$

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## 6.2. RESULTS

## 6.2.1. ISOLATION OF Flk-1

To identify RTKs that are expressed during mouse development, PCR assays using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of RTKs were performed (Hanks, S.K. et al. 1988, Science 241:42-52). DNA extracted from a  $\lambda$ gt10 cDNA library of day 8.5 mouse embryos (Fahrner, K. et al.,

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-43-

1987, EMBO. J., 6:1497-1508), a stage in mouse development at which many differentiation processes begin was used as the template in the PCR assays. In a parallel approach, with the intention of identifying RTKs that regulate angiogenesis, similar primers were used for the amplification of RTK cDNA sequences from capillary endothelial cells that had been isolated from the brains of postnatal day 4-8 mice, a time at which brain endothelial cell proliferation is maximal (Robertson, P.L. et al., 1985, Devel. Brain Res. 23:219-223). Both approaches yielded cDNA sequences (FIG. 11, SEQ. ID NO.:) encoding the recently described fetal liver RTK, Flk-1 (Matthews, W. et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich, A. and Schlessinger, J. 1990, Cell 61:203-212) and is closely related to human *flt*, which also contains seven immunoglobulin-like repeats in its extracellular domain in contrast to other RTKs of that subfamily, which contain only five such repeat structures (Matthews, W. et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030). Sequence comparisons of Flk-1 with KDR (Terman, B.I. et al., 1991, Oncogene 6:1677-1683) and TKr-C (Sarzani, R. et al., 1992, Biochem. Biophys. Res. Comm. 186:706-714) suggest that these are the human and rat homologues of Flk-1, respectively (Figure 1).

#### 6.2.2 EXPRESSION OF Flk-1 mRNA DURING EMBRYONIC DEVELOPMENT

As a first step towards the elucidation of the biological function of Flk-1, the expression of Flk-1 mRNA was analyzed in mouse embryos at different development stages. Northern blot hybridization experiments indicated abundant expression of a major 5.5 kb mRNA between day 9.5 and day 18.5, with an apparent

-44-

decline towards the end of gestation (Figure 2A). In postnatal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain mRNA (Figure 2B).

5        In situ hybridization experiments were performed to obtain more detailed information about the expression of Flk-1 during different embryonal stages. A single-stranded antisense, 2619-nucleotide-long DNA probe comprising the Flk-1 extracellular domain was used as a  
10       probe because it generated the most specific hybridization signals. As an example, a parasagittal section of a day 14.5 embryo is shown in Figure 3. High levels of hybridization were detected in the ventricle of the heart, the lung, and the meninges; other tissues such  
15       as brain, liver, and mandible appeared to contain fewer cells expressing Flk-1 mRNA. Thin strands of Flk-1 expression were also observed in the intersegmental regions of the vertebrae and at the inner surface of the atrium and the aorta. Higher magnification revealed that  
20       the expression of Flk-1 seemed to be restricted to capillaries and blood vessels. Closer examination of the heart, for example, showed positive signals only in the ventricular capillaries and endothelial lining of the atrium (Figure 4A). In the lung, Flk-1 expression was  
25       detected in peribronchial capillaries, but was absent from bronchial epithelium (Figure 4D). The aorta showed strong hybridization in endothelial cells, but not in the muscular layer (Figure 4C).

30       6.2.3. EXPRESSION OF Flk-1 DURING ORGAN ANGIOGENESIS

      The neuroectoderm in the telencephalon of a day 11.5 mouse embryo is largely avascular; the first vascular sprouts begin to radially invade the organ originating from the perineural vascular plexus (Bär, J., 1980, Adv.  
35       Anat. Embryol. Cell. Biol. 59:1-62; Risau, W. and Lemmon,

-45-

V. 1988, Dev. Biol. 125:441-450). At this stage, expression of Flk-1 was high in the perineural vascular plexus and in invading vascular sprouts, as shown in Figure 5A. These in situ hybridization analyses indicated that the proliferating endothelial cells of an angiogenic sprout expressed the Flk-1 mRNA. At day 14.5, when the neuroectoderm is already highly vascularized, numerous radial vessels as well as branching vessels of the intraneural plexus contained large amounts of Flk-1 mRNA (Figure 5B). At postnatal day 4, when sprouting and endothelial cell proliferation is at its highest, strong expression of Flk-1 mRNA was observed in endothelial cells (Figure 5C). Conversely, in the adult brain when angiogenesis has ceased, Flk-1 expression was very low (Figure 5D) and appeared to be restricted mainly to the choroid plexus (Figure 6). In the choroid plexus, cells in the inner vascular layer expressed Flk-1 mRNA, while epithelial cells did not (Figure 6A, B).

The embryonic kidney is vascularized by an angiogenic process (Ekblom, P. et al., 1982, Cell Diff. 11:35-39). Glomerular and peritubular capillaries develop synchronously with epithelial morphogenesis. In the postnatal day 4 kidney, in addition to other capillaries, prominent expression of Flk-1 was observed in the presumptive glomerular capillaries (Figure 7A). This expression persisted in the adult kidney (Figure 7C and D) and then seemed to be more confined to the glomerular compared to the early postnatal kidney.

#### 6.2.4. Flk-1 EXPRESSION IN ENDOTHELIAL CELL PROGENITORS

To investigate the possible involvement of Flk-1 in the early stages of vascular development, analysis of embryos at different stages during blood island formation were performed. In a sagittal section of the deciduum of

-46-

a day 8.5 mouse embryo, Flk-1 expression was detected on maternal blood vessels in the deciduum, in the yolk sac and in the trophectoderm. Flk-1 mRNA was also found in the allantois and inside the embryo, mainly located in that part where mesenchyma is found (Figure 8A). At a higher magnification of the maternal deciduum, high levels of Flk-1 mRNA expression were found in the inner lining of blood vessels, which consist of endothelial cells (Figure 8B). In the yolk sac, hybridization signals were confined to the mesodermal layer, in which the hemangioblasts differentiate (Figure 8C). Figure 8D shows a blood island at higher magnification, in which the peripheral angioblasts expressed a high level of Flk-1 mRNA.

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#### 6.2.5. Flk-1 IS A HIGH AFFINITY RECEPTOR FOR VEGF

Detailed examination of in situ hybridization results and comparison with those for VEGF recently reported by Breier, G. et al. (1992, Development 114:521-532) revealed a remarkable similarity in expression pattern. Furthermore, Flk-1 expression in the glomerular endothelium and VEGF in the surrounding epithelial cells (Breier, G. et al., 1992, Development 114:521-532) raised the possibility of a paracrine relationship between these cells types and suggested therefore a ligand-receptor relationship for VEGF and Flk-1, respectively. In order to test this hypothesis, the full-length Flk-1 cDNA was cloned into the mammalian expression vector pCMV, which contains transcriptional control elements of the human cytomegalovirus (Gorman, C.M. et al., 1989, Virology 171:377-385). For transient expression of the receptor, the Flk-1 expressing plasmid was then transfected into COS-1 fibroblasts.

Specific binding of VEGF to the Flk-1 RTK was demonstrated by crosslinking and competition binding.

-47-

experiments. Purified  $^{125}\text{I}$ -labeled VEGF was incubated with COS-1 cells transfected with the pCMV-Flk-1 expression vector. Crosslinking with DSS and subsequent analysis of immunoprecipitation, PAGE, and autoradiography revealed an approximately 220 kD band which was not detected in the control experiment with untransfected COS-1 cells and is likely to represent the VEGF/Flk-1 receptor complex (Figure 9A). In addition, VEGF competed with  $^{125}\text{I}$ -VEGF binding to Flk-1 expressing COS-1 cells (Figure 9B), whereas untransfected COS-1 cells did not bind  $^{125}\text{I}$ -VEGF. The interaction of VEGF with the receptor on transfected cells was specific, as PDGF-BB did not compete with binding of  $^{125}\text{I}$ -VEGF. Analysis of the binding data revealed a Kd of about  $10^{-10}$  M, suggesting that Flk-1 is a high affinity receptor of VEGF. This finding, together with the Flk-1 and VEGF in situ hybridization results strongly suggests that Flk-1 is a physiologically relevant receptor for VEGF.

An autophosphorylation assay was performed to confirm the biological relevance of VEGF binding to the Flk-1 receptor. COS1 cells which transiently expressed Flk-1 were starved in DMEM containing 0.5% fetal calf serum for 24h, stimulated with 0.5 mM VEGF, and lysed. The receptors were immunoprecipitated with the Flk-1 specific polyclonal antibody CT128, and then analyzed by SDS-PAGE and subsequent immunoblotting using the antiphosphotyrosine antibody 5E2 (Fendly, B.M. et al., 1990, Cancer Research 50:1550-1558). As shown in Figure 10, VEGF stimulation of Flk-1 expressing cells led to a significant induction of tyrosine phosphorylation of the 180 kD Flk-1 receptor.

-48-

6.2.6. INHIBITION OF TUMOR GROWTH BY  
TRANSDOMINANT-NEGATIVE INHIBITION OF Flk-1

The Flk-1 receptor is believed to play a major role in vasculogenesis and angiogenesis. Therefore, inhibition of Flk-1 activity may inhibit vasculogenesis of a developing tumor and inhibit its growth. To test this hypothesis, tumor cells (C6 rat glioblastoma) and mouse cells producing a recombinant retrovirus encoding a truncated Flk-1 receptor were mixed and implanted subcutaneously into nude mice. The implanted C6 glioblastoma cells secrete VEGF which will bind to and activate the Flk-1 receptors expressed on the surface of mouse endothelial cells. In the absence of any inhibitors of vasculogenesis, the endothelial cells will proliferate and migrate towards the tumor cells. Alternatively, if at the time of injection, the tumor cells are co-injected with cells producing recombinant retrovirus encoding the dominant-negative Flk-1, the endothelial cells growing towards the implanted tumor cells will become infected with recombinant retrovirus which may result in dominant-negative Flk-1 mutant expression and inhibition of endogenous Flk-1 signaling. Suppression of endothelial cell proliferation and migration will result in failure of the implanted tumor cells to become vascularized which will lead to inhibition of tumor growth. As shown in Figures 12 and 13, tumor growth is significantly inhibited in mice receiving implantations of cells producing truncated Flk-1 indicating that expression of a truncated Flk-1 receptor can act in a dominant-negative manner to inhibit the activity of endogenous wild-type Flk-1.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are

-49-

functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the  
5 foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for  
10 purposes of description.

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-50-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Ullrich, et al
- (ii) TITLE OF INVENTION: FIX-1 IS A RECEPTOR FOR VASCULAR  
ENDOTHELIAL GROWTH FACTOR
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Pennie & Edmonds
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  - (C) CITY: New York
  - (D) STATE: New York
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 10036-2711
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: To be assigned
  - (B) FILING DATE: 03-MAR-1993
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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  - (B) REGISTRATION NUMBER: 30,742
  - (C) REFERENCE/DOCKET NUMBER: 7683-034-999
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  - (B) TELEFAX: (212) 869-8864/9741
  - (C) TELEX: 66141 PENNIE

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5470 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 286..4386

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGAATTCGGG CCCAGACTGT GTCCCGCAGC CGGGATAACC TGGCTGACCC GATTCCGCGG	120
ACACCGCTGA CAGCCGCGGC TGGAGCCAGG GCGCCGGTGC CCCGCGCTCT CCCCGGTCTT	180
GCGCTGCGGG GGCCATACCG CCTCTGTGAC TTCTTTGCCG GCCAGGGACG GAGAAGGAGT	240



-51-

CTGTGCCTGA GAAACTGGGC TCTGTGCCCA GGCGCGAGGT GCAGG ATG GAG AGC	294
Met Glu Ser	
1	
AAG GCG CTG CTA GCT GTC GCT CTG TGG TTC TGC GTG GAG ACC CGA GCC	342
Lys Ala Leu Leu Ala Val Ala Leu Trp Phe Cys Val Glu Thr Arg Ala	
5 10 15	
GCC TCT GTG GGT TTG ACT GGC GAT TTT CTC CAT CCC CCC AAG CTC AGC	390
Ala Ser Val Gly Leu Thr Gly Asp Phe Leu His Pro Pro Lys Leu Ser	
20 25 30 35	
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Thr Gln Lys Asp Ile Leu Thr Ile Leu Ala Asn Thr Thr Leu Gln Ile	
40 45 50	
ACT TGC AGG CGA CAG CGG GAC CTG GAC TGG CTT TGG CCC AAT GCT CAG	486
Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro Asn Ala Gln	
55 60 65	
CGT GAT TCT GAG GAA AGG GTA TTG GTG ACT GAA TGC GGC GGT GGT GAC	534
Arg Asp Ser Glu Glu Arg Val Leu Val Thr Glu Cys Gly Gly Gly Asp	
70 75 80	
AGT ATC TTC TGC AAA ACA CTC ACC ATT CCC AGG GTG GTT GGA AAT GAT	582
Ser Ile Phe Cys Lys Thr Leu Thr Ile Pro Arg Val Val Gly Asn Asp	
85 90 95	
ACT GGA GCC TAC AAG TGC TCG TAC CGG GAC GTC GAC ATA GCC TCC ACT	630
Thr Gly Ala Tyr Lys Cys Ser Tyr Arg Asp Val Asp Ile Ala Ser Thr	
100 105 110 115	
GTT TAT GTC TAT GTT CGA GAT TAC AGA TCA CCA TTC ATC GCC TCT GTC	678
Val Tyr Val Tyr Val Arg Asp Tyr Arg Ser Pro Phe Ile Ala Ser Val	
120 125 130	
AGT GAC CAG CAT GGC ATC GTG TAC ATC ACC GAG AAC AAG AAC AAA ACT	726
Ser Asp Gln His Gly Ile Val Tyr Ile Thr Glu Asn Lys Asn Lys Thr	
135 140 145	
GTG GTG ATC CCC TGC CGA GGG TCG ATT TCA AAC CTC AAT GTG TCT CTT	774
Val Val Ile Pro Cys Arg Gly Ser Ile Ser Asn Leu Asn Val Ser Leu	
150 155 160	
TGC GCT AGG TAT CCA GAA AAG AGA TTT GTT CCG GAT GGA AAC AGA ATT	822
Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly Asn Arg Ile	
165 170 175	
TCC TGG GAC AGC GAG ATA GGC TTT ACT CTC CCC AGT TAC ATG ATC AGC	870
Ser Trp Asp Ser Glu Ile Gly Phe Thr Leu Pro Ser Tyr Met Ile Ser	
180 185 190 195	
TAT GCC GGC ATG GTC TTC TGT GAG GCA AAG ATC AAT GAT GAA ACC TAT	918
Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile Asn Asp Glu Thr Tyr	
200 205 210	
CAG TCT ATC ATG TAC ATA GTT GTG GTT GTA GGA TAT AGG ATT TAT GAT	966
Gln Ser Ile Met Tyr Ile Val Val Val Val Gly Tyr Arg Ile Tyr Asp	
215 220 225	
GTG ATT CTG AGC CCC CCG CAT GAA ATT GAG CTA TCT GCC GGA GAA AAA	1014
Val Ile Leu Ser Pro Pro His Glu Ile Glu Leu Ser Ala Gly Glu Lys	
230 235 240	
CTT GTC TTA AAT TGT ACA GCG AGA ACA GAG CTC AAT GTG GGG CTT GAT	1062
Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Leu Asp	
245 250 255	

-52-

TTC ACC TGG CAC TCT CCA CCT TCA AAG TCT CAT CAT AAG AAG ATT GTA	1110
Phe Thr Trp His Ser Pro Pro Ser Lys Ser His His Lys Lys Ile Val	
260 265 270 275	
AAC CGG GAT GTG AAA CCC TTT CCT GCG ACT GTG CCG AAG ATG TTT TTG	1158
Asn Arg Asp Val Lys Pro Phe Pro Gly Thr Val Ala Lys Met Phe Leu	
280 285 290	
AGC ACC TTG ACA ATA GAA AGT GTG ACC AAG AGT GAC CAA GGG GAA TAC	1206
Ser Thr Leu Thr Ile Glu Ser Val Thr Lys Ser Asp Gln Gly Glu Tyr	
295 300 305	
ACC TGT GTA GCG TCC AGT GGA CCG ATG ATC AAG AGA AAT AGA ACA TTT	1254
Thr Cys Val Ala Ser Ser Gly Arg Met Ile Lys Arg Asn Arg Thr Phe	
310 315 320	
GTC CGA GTT CAC ACA AAG CCT TTT ATT GCT TTC GGT AGT GGG ATG AAA	1302
Val Arg Val His Thr Lys Pro Phe Ile Ala Phe Gly Ser Gly Met Lys	
325 330 335	
TCT TTG GTG GAA GCC ACA GTG GGC AGT CAA GTC CGA ATC CCT GTG AAG	1350
Ser Leu Val Glu Ala Thr Val Gly Ser Gln Val Arg Ile Pro Val Lys	
340 345 350 355	
TAT CTC AGT TAC CCA GCT CCT GAT ATC AAA TGG TAC AGA AAT GGA AGG	1398
Tyr Leu Ser Tyr Pro Ala Pro Asp Ile Lys Trp Tyr Arg Asn Gly Arg	
360 365 370	
CCC ATT GAG TCC AAC TAC ACA ATG ATT GTT GGC GAT GAA CTC ACC ATC	1446
Pro Ile Glu Ser Asn Tyr Thr Met Ile Val Gly Asp Glu Leu Thr Ile	
375 380 385	
ATG GAA GTG ACT GAA AGA GAT GCA GGA AAC TAC ACG GTC ATC CTC ACC	1494
Met Glu Val Thr Glu Arg Asp Ala Gly Asn Tyr Thr Val Ile Leu Thr	
390 395 400	
AAC CCC ATT TCA ATG GAG AAA CAG AGC CAC ATG GTC TCT CTG GTT GTG	1542
Asn Pro Ile Ser Met Glu Lys Gln Ser His Met Val Ser Leu Val Val	
405 410 415	
AAT GTC CCA CCC CAG ATC GGT GAG AAA GCC TTG ATC TCG CCT ATG GAT	1590
Asn Val Pro Pro Gln Ile Gly Glu Lys Ala Leu Ile Ser Pro Met Asp	
420 425 430 435	
TCC TAC CAG TAT GGG ACC ATG CAG ACA TTG ACA TGC ACA GTC TAC GCC	1638
Ser Tyr Gln Tyr Gly Thr Met Gln Thr Leu Thr Cys Thr Val Tyr Ala	
440 445 450	
AAC CCT CCC CTG CAC CAC ATC CAG TGG TAC TGG CAG CTA GAA GAA GCC	1686
Asn Pro Pro Leu His His Ile Gln Trp Tyr Trp Gln Leu Glu Glu Ala	
455 460 465	
TGC TCC TAC AGA CCC GGC CAA ACA AGC CCG TAT GCT TGT AAA GAA TGG	1734
Cys Ser Tyr Arg Pro Gly Gln Thr Ser Pro Tyr Ala Cys Lys Glu Trp	
470 475 480	
AGA CAC GTG GAG GAT TTC CAG GGG GGA AAC AAG ATC GAA GTC ACC AAA	1782
Arg His Val Glu Asp Phe Gln Gly Gly Asn Lys Ile Glu Val Thr Lys	
485 490 495	
AAC CAA TAT GCC CTG ATT GAA GGA AAA AAC AAA ACT GTA AGT ACG CTG	1830
Asn Gln Tyr Ala Leu Ile Glu Gly Lys Asn Lys Thr Val Ser Thr Leu	
500 505 510 515	
GTC ATC CAA GCT GCC AAC GTG TCA GCG TTG TAC AAA TGT GAA GCC ATC	1878
Val Ile Gln Ala Ala Asn Val Ser Ala Leu Tyr Lys Cys Glu Ala Ile	
520 525 530	

-53-

AAC AAA GCG GGA CGA GGA GAG AGG GTC ATC TCC TTC CAT GTG ATC AGG Asn Lys Ala Gly Arg Gly Glu Arg Val Ile Ser Phe His Val Ile Arg 535 540 545	1926
GGT CCT GAA ATT ACT GTG CAA CCT GCT GCC CAG CCA ACT GAG CAG GAG Gly Pro Glu Ile Thr Val Gln Pro Ala Ala Gln Pro Thr Glu Gln Glu 550 555 560	1974
AGT GTG TCC CTG TTG TGC ACT GCA GAC AGA AAT ACG TTT GAG AAC CTC Ser Val Ser Leu Leu Cys Thr Ala Asp Arg Asn Thr Phe Glu Asn Leu 565 570 575	2022
ACG TGG TAC AAG CTT GGC TCA CAG GCA ACA TCG GTC CAC ATG GGC GAA Thr Trp Tyr Lys Leu Gly Ser Gln Ala Thr Ser Val His Met Gly Glu 580 585 590 595	2070
TCA CTC ACA CCA GTT TGC AAG AAC TTG GAT GCT CTT TGG AAA CTG AAT Ser Leu Thr Pro Val Cys Lys Asn Leu Asp Ala Leu Trp Lys Leu Asn 600 605 610	2118
GGC ACC ATG TTT TCT AAC AGC ACA AAT GAC ATC TTG ATT GTG GCA TTT Gly Thr Met Phe Ser Asn Ser Thr Asn Asp Ile Leu Ile Val Ala Phe 615 620 625	2166
CAG AAT GCC TCT CTG CAG GAC CAA GGC GAC TAT GTT TGC TCT GCT CAA Gln Asn Ala Ser Leu Gln Asp Gln Gly Asp Tyr Val Cys Ser Ala Gln 630 635 640	2214
GAT AAG AAG ACC AAG AAA AGA CAT TGC CTG GTC AAA CAG CTC ATC ATC Asp Lys Lys Thr Lys Lys Arg His Cys Leu Val Lys Gln Leu Ile Ile 645 650 655	2262
CTA GAG CGC ATG GCA CCC ATG ATC ACC GGA AAT CTG GAG AAT CAG ACA Leu Glu Arg Met Ala Pro Met Ile Thr Gly Asn Leu Glu Asn Gln Thr 660 665 670 675	2310
ACA ACC ATT GGC GAG ACC ATT GAA GTG ACT TGC CCA GCA TCT GGA AAT Thr Thr Ile Gly Glu Thr Ile Glu Val Thr Cys Pro Ala Ser Gly Asn 680 685 690	2358
CCT ACC CCA CAC ATT ACA TGG TTC AAA GAC AAC GAG ACC CTG GTA GAA Pro Thr Pro His Ile Thr Trp Phe Lys Asp Asn Glu Thr Leu Val Glu 695 700 705	2406
GAT TCA GGC ATT GTA CTG AGA GAT GGG AAC CGG AAC CTG ACT ATC CGC Asp Ser Gly Ile Val Leu Arg Asp Gly Asn Arg Asn Leu Thr Ile Arg 710 715 720	2454
AGG GTG AGG AAG GAG GAT GGA GGC CTC TAC ACC TGC CAG GCC TGC AAT Arg Val Arg Lys Glu Asp Gly Gly Leu Tyr Thr Cys Gln Ala Cys Asn 725 730 735	2502
GTC CTT GGC TGT GCA AGA GCG GAG ACG CTC TTC ATA ATA GAA GGT GCC Val Leu Gly Cys Ala Arg Ala Glu Thr Leu Phe Ile Ile Glu Gly Ala 740 745 750 755	2550
CAG GAA AAG ACC AAC TTG GAA GTC ATT ATC CTC GTC GGC ACT GCA GTG Gln Glu Lys Thr Asn Leu Glu Val Ile Ile Leu Val Gly Thr Ala Val 760 765 770	2598
ATT GCC ATG TTC TTC TGG CTC CTT CTT GTC ATT GTC CTA CGG ACC GTT Ile Ala Met Phe Phe Trp Leu Leu Leu Val Ile Val Leu Arg Thr Val 775 780 785	2646
AAG CGG GCC AAT GAA GGG GAA CTG AAG ACA GGC TAC TTG TCT ATT GTC Lys Arg Ala Asn Glu Gly Glu Leu Lys Thr Gly Tyr Leu Ser Ile Val 790 795 800	2694

-54-

ATG Met	GAT Asp	CCA Pro	GAT Asp	GAA Glu	TTG Leu	CCC Pro	TTG Leu	GAT Asp	GAG Glu	CGC Arg	TGT Cys	GAA Glu	CGC Arg	TTG Leu	CCT Pro	2742
805						810					815					
TAT Tyr	GAT Asp	GCC Ala	AGC Ser	AAG Lys	TGG Trp	GAA Glu	TTC Phe	CCC Pro	AGG Arg	GAC Asp	CGG Arg	CTG Leu	AAA Lys	CTA Leu	GGA Gly	2790
820					825					830					835	
AAA Lys	CCT Pro	CTT Leu	GGC Gly	CGC Arg	GGT Gly	GCC Ala	TTC Phe	GGC Gly	CAA Gln	GTG Val	ATT Ile	GAG Glu	GCA Ala	GAC Asp	GCT Ala	2838
				840					845					850		
TTT Phe	GGA Gly	ATT Ile	GAC Asp	AAG Lys	ACA Thr	GCG Ala	ACT Thr	TGC Cys	AAA Lys	ACA Thr	GTA Val	GCC Ala	GTC Val	AAG Lys	ATG Met	2886
			855					860					865			
TTG Leu	AAA Lys	GAA Glu	GGA Gly	GCA Ala	ACA Thr	CAC His	AGC Ser	GAG Glu	CAT His	CGA Arg	GCC Ala	CTC Leu	ATG Met	TCT Ser	GAA Glu	2934
		870					875					880				
CTC Leu	AAG Lys	ATC Ile	CTC Leu	ATC Ile	CAC His	ATT Ile	GGT Gly	CAC His	CAT His	CTC Leu	AAT Asn	GTG Val	GTG Val	AAC Asn	CTC Leu	2982
	885					890					895					
CTA Leu	GGC Gly	GCC Ala	TGC Cys	ACC Thr	AAG Lys	CCG Pro	GGA Gly	GGG Gly	CCT Pro	CTC Leu	ATG Met	GTG Val	ATT Ile	GTG Val	GAA Glu	3030
900					905					910					915	
TTC Phe	TGC Cys	AAG Lys	TTT Phe	GGA Gly	AAC Asn	CTA Leu	TCA Ser	ACT Thr	TAC Tyr	TTA Leu	CGG Arg	GGC Gly	AAG Lys	AGA Arg	AAT Asn	3078
				920					925					930		
GAA Glu	TTT Phe	GTT Val	CCC Pro	TAT Tyr	AAG Lys	AGC Ser	AAA Lys	GGG Gly	GCA Ala	CGC Arg	TTC Phe	CGC Arg	CAG Gln	GGC Gly	AAG Lys	3126
			935					940					945			
GAC Asp	TAC Tyr	GTT Val	GGG Gly	GAG Glu	CTC Leu	TCC Ser	GTG Val	GAT Asp	CTG Leu	AAA Lys	AGA Arg	CGC Arg	TTG Leu	GAC Asp	AGC Ser	3174
		950					955					960				
ATC Ile	ACC Thr	ACC Ser	AGC Ser	CAG Gln	AGC Ser	TCT Ser	GCC Ala	AGC Ser	TCA Ser	GGC Gly	TTT Phe	GTT Val	GAG Glu	GAG Glu	AAA Lys	3222
	965					970					975					
TCG Ser	CTC Leu	AGT Ser	GAT Asp	GTA Val	GAG Glu	GAA Glu	GAA Glu	GAA Glu	GCT Ala	TCT Ser	GAA Glu	GAA Glu	CTG Leu	TAC Tyr	AAG Lys	3270
980					985				990						995	
GAC Asp	TTC Phe	CTG Leu	ACC Thr	TTG Leu	GAG Glu	CAT His	CTC Leu	ATC Ile	TGT Cys	TAC Tyr	AGC Ser	TTC Phe	CAA Gln	GTG Val	GCT Ala	3318
				1000					1005					1010		
AAG Lys	GGC Gly	ATG Met	GAG Glu	TTC Phe	TTG Leu	GCA Ala	TCA Ser	AGG Arg	AAG Lys	TGT Cys	ATC Ile	CAC His	AGG Arg	GAC Asp	CTG Leu	3366
			1015					1020					1025			
GCA Ala	GCA Ala	CGA Arg	AAC Asn	ATT Ile	CTC Leu	CTA Leu	TCG Ser	GAG Glu	AAG Lys	AAT Asn	GTG Val	GTT Val	AAG Lys	ATC Ile	TGT Cys	3414
		1030					1035					1040				
GAC Asp	TTC Phe	GGC Gly	TTG Leu	GCC Ala	CGG Arg	GAC Asp	ATT Ile	TAT Tyr	AAA Lys	GAC Asp	CCG Pro	GAT Asp	TAT Tyr	GTC Val	AGA Arg	3462
	1045					1050				1055						
AAA Lys	GGA Gly	GAT Asp	GCC Ala	CGA Arg	CTC Leu	CCT Pro	TTG Leu	AAG Lys	TGG Trp	ATC Met	GCC Ala	CCG Pro	GAA Glu	ACC Thr	ATT Ile	3510
1060					1065					1070					1075	

SUBSTITUTE SHEET

-55-

TTT GAC ACA GTA TAC ACA ATT CAG AGC GAT GTG TGG TCT TTC GGT GTG Phe Asp Arg Val Tyr Thr Ile Gln Ser Asp Val Trp Ser Phe Gly Val 1080 1085 1090	3558
TTG CTC TGG GAA ATA TTT TCC TTA GGT GCC TCC CCA TAC CCT GGG GTC Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr Pro Gly Val 1095 1100 1105	3606
AAG ATT GAT GAA GAA TTT TGT AGG AGA TTG AAA GAA GGA ACT AGA ATG Lys Ile Asp Glu Glu Phe Cys Arg Arg Leu Lys Glu Gly Thr Arg Met 1110 1115 1120	3654
CGG GCT CCT GAC TAC ACT ACC CCA GAA ATG TAC CAG ACC ATG CTG GAC Arg Ala Pro Asp Tyr Thr Thr Pro Glu Met Tyr Gln Thr Met Leu Asp 1125 1130 1135	3702
TGC TGG CAT GAG GAC CCC AAC CAG AGA CCC TCG TTT TCA GAG TTG GTG Cys Trp His Glu Asp Pro Asn Gln Arg Pro Ser Phe Ser Glu Leu Val 1140 1145 1150 1155	3750
GAG CAT TTG GGA AAC CTC CTG CAA GCA AAT GCG CAG CAG GAT GGC AAA Glu His Leu Gly Asn Leu Leu Gln Ala Asn Ala Gln Gln Asp Gly Lys 1160 1165 1170	3798
GAC TAT ATT GTT CTT CCA ATG TCA GAG ACA CTG AGC ATG GAA GAG GAT Asp Tyr Ile Val Leu Pro Met Ser Glu Thr Leu Ser Met Glu Glu Asp 1175 1180 1185	3846
TCT GGA CTC TCC CTG CCT ACC TCA CCT GTT TCC TGT ATG GAG GAA GAG Ser Gly Leu Ser Leu Pro Thr Ser Pro Val Ser Cys Met Glu Glu Glu 1190 1195 1200	3894
GAA GTG TGC GAC CCC AAA TTC CAT TAT GAC AAC ACA GCA GGA ATC AGT Glu Val Cys Asp Pro Lys Phe His Tyr Asp Asn Thr Ala Gly Ile Ser 1205 1210 1215	3942
CAT TAT CTC CAG AAC AGT AAG CGA AAG AGC CGG CCA GTG AGT GTA AAA His Tyr Leu Gln Asn Ser Lys Arg Lys Ser Arg Pro Val Ser Val Lys 1220 1225 1230 1235	3990
ACA TTT GAA GAT ATC CCA TTG GAG GAA CCA GAA GTA AAA GTG ATC CCA Thr Phe Glu Asp Ile Pro Leu Glu Glu Pro Glu Val Lys Val Ile Pro 1240 1245 1250	4038
GAT GAC AGC CAG ACA GAC AGT GGG ATG GTC CTT GCA TCA GAA GAG CTG Asp Asp Ser Gln Thr Asp Ser Gly Met Val Leu Ala Ser Glu Glu Leu 1255 1260 1265	4086
AAA ACT CTG GAA GAC AGG AAC AAA TTA TCT CCA TCT TTT GGT GGA ATG Lys Thr Leu Glu Asp Arg Asn Lys Leu Ser Pro Ser Phe Gly Gly Met 1270 1275 1280	4134
ATG CCC AGT AAA AGC AGG GAG TCT GTG GCC TCG GAA GGC TCC AAC CAG Met Pro Ser Lys Ser Arg Glu Ser Val Ala Ser Glu Gly Ser Asn Gln 1285 1290 1295	4182
ACC AGT GGC TAC CAG TCT GGG TAT CAC TCA GAT GAC ACA GAC ACC ACC Thr Ser Gly Tyr Gln Ser Gly Tyr His Ser Asp Asp Thr Asp Thr Thr 1300 1305 1310 1315	4230
GTG TAC TCC AGC GAC GAG GCA GGA CTT TTA AAG ATG GTG GAT GCT GCA Val Tyr Ser Ser Asp Glu Ala Gly Leu Leu Lys Met Val Asp Ala Ala 1320 1325 1330	4278
GTT CAC GCT GAC TCA GGG ACC ACA CTG CAG CTC ACC TCC TGT TTA AAT Val His Ala Asp Ser Gly Thr Thr Leu Gln Leu Thr Ser Cys Leu Asn 1335 1340 1345	4326

-56-

CGA AGT GGT CCT GTC CCG GCT CCG CCC CCA ACT CCT GGA AAT CAC GAG	4374
Gly Ser Gly Pro Val Pro Ala Pro Pro Pro Thr Pro Gly Asn His Glu	
1350 1355 1360	
AGA GGT GCT GCT TAGATTTTCA AGTGTGTGTTT TTTCCACCAC CCGGAAGTAG	4426
Arg Gly Ala Ala	
1365	
CCACATTTGA TTTTCATTTT TGGAGGAGGG ACCTCAGACT GCAAGGAGCT TGTCTCAGG	4486
GCATTTCCAG AGAAGATGCC CATGACCCAA GAATGTGTG ACTCTACTCT CTTTTCATT	4546
CATTTAAAG TCCTATATAA TGTGCCCTGC TGTGGTCTCA CTACCAGTTA AAGCAAAGA	4606
CTTTCAAACA CGTGGACTCT GTCTCCAAG AAGTGGCAAC GGCACCTCTG TGAACTGGA	4666
TCGAATGGGC AATGCTTTGT GTGTGAGGA TGGGTGAGAT GTCCCAGGGC CGAGTCTGTC	4726
TACCTTGGAG GCTTTGTGGA GGATGCGGGC TATGAGCCAA GTGTTAAGTG TGGGATGTGG	4786
ACTGGGAGGA AGGAAGGCGC AAGTCGCTCG GAGAGCGGTT GGAGCCTGCA GATGCATTGT	4846
GCTGGCTCTG GTGGAGGTGG GCTTGTGGCC TGTGAGGAAA CGCAAGGCG GCGGGCAGGG	4906
TTTGGTTTTG GAAGGTTTGC GTGCTCTTCA CAGTCGGGTT ACAGGCGAGT TCCCTGTGGC	4966
GTTTCTACT CCTAATGAGA GTTCCTTCCG GACTCTTACG TGTCTCCTGG CCTGGCCCCA	5026
GGAAGGAAAT GATGCAGCTT GCTCCTTCCT CATCTCTCAG GCTGTGCCTT AATTCAGAAC	5086
ACCAAAGAG AGGAACGTCG GCAGAGGCTC CTGACGGGGC CGAAGAATTG TGAGAACAGA	5146
ACAGAACTC AGGGTTTCTG CTGGGTGGAG ACCCACGTGG CGCCCTGGTG GCAGGTCTGA	5206
GGGTTCTCTG TCAAGTGGCG GTAAAGGCTC AGGCTGGTGT TCTTCTCTA TCTCCACTCC	5266
TGTCAGGCCC CCAAGTCCTC AGTATTTTAG CTTTGTGGCT TCCTGATGGC AGAAAAATCT	5326
TAATTGGTTG GTTTGCTCTC CAGATAATCA CTAGCCAGAT TTCGAAATTA CTTTTTAGCC	5386
GAGGTTATGA TAACATCTAC TGTATCCTTT AGAATTTTAA CCTATAAAC TATGTCTACT	5446
GGTTTCTGCC TGTGTGCTTA TGTT	5470

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1367 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Glu	Ser	Lys	Ala	Leu	Leu	Ala	Val	Ala	Leu	Trp	Phe	Cys	Val	Glu
1				5				10						15	
Thr	Arg	Ala	Ala	Ser	Val	Gly	Leu	Thr	Gly	Asp	Phe	Leu	His	Pro	Pro
		20						25					30		
Lys	Leu	Ser	Thr	Gln	Lys	Asp	Ile	Leu	Thr	Ile	Leu	Ala	Asn	Thr	Thr
		35					40					45			
Leu	Gln	Ile	Thr	Cys	Arg	Gly	Gln	Arg	Asp	Leu	Asp	Trp	Leu	Trp	Pro
	50					55				60					

-57-

Asn Ala Gln Arg Asp Ser Glu Glu Arg Val Leu Val Thr Glu Cys Gly  
 65 70 75 80  
 Gly Gly Asp Ser Ile Phe Cys Lys Thr Leu Thr Ile Pro Arg Val Val  
 85 90 95  
 Gly Asn Asp Thr Gly Ala Tyr Lys Cys Ser Tyr Arg Asp Val Asp Ile  
 100 105 110  
 Ala Ser Thr Val Tyr Val Tyr Val Arg Asp Tyr Arg Ser Pro Phe Ile  
 115 120 125  
 Ala Ser Val Ser Asp Gln His Gly Ile Val Tyr Ile Thr Glu Asn Lys  
 130 135 140  
 Asn Lys Thr Val Val Ile Pro Cys Arg Gly Ser Ile Ser Asn Leu Asn  
 145 150 155 160  
 Val Ser Leu Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly  
 165 170 175  
 Asn Arg Ile Ser Trp Asp Ser Glu Ile Gly Phe Thr Leu Pro Ser Tyr  
 180 185 190  
 Met Ile Ser Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile Asn Asp  
 195 200 205  
 Glu Thr Tyr Gln Ser Ile Met Tyr Ile Val Val Val Val Gly Tyr Arg  
 210 215 220  
 Ile Tyr Asp Val Ile Leu Ser Pro Pro His Glu Ile Glu Leu Ser Ala  
 225 230 235 240  
 Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val  
 245 250 255  
 Gly Leu Asp Phe Thr Trp His Ser Pro Pro Ser Lys Ser His His Lys  
 260 265 270  
 Lys Ile Val Asn Arg Asp Val Lys Pro Phe Pro Gly Thr Val Ala Lys  
 275 280 285  
 Met Phe Leu Ser Thr Leu Thr Ile Glu Ser Val Thr Lys Ser Asp Gln  
 290 295 300  
 Gly Glu Tyr Thr Cys Val Ala Ser Ser Gly Arg Met Ile Lys Arg Asn  
 305 310 315 320  
 Arg Thr Phe Val Arg Val His Thr Lys Pro Phe Ile Ala Phe Gly Ser  
 325 330 335  
 Gly Met Lys Ser Leu Val Glu Ala Thr Val Gly Ser Gln Val Arg Ile  
 340 345 350  
 Pro Val Lys Tyr Leu Ser Tyr Pro Ala Pro Asp Ile Lys Trp Tyr Arg  
 355 360 365  
 Asn Gly Arg Pro Ile Glu Ser Asn Tyr Thr Met Ile Val Gly Asp Glu  
 370 375 380  
 Leu Thr Ile Met Glu Val Thr Glu Arg Asp Ala Gly Asn Tyr Thr Val  
 385 390 395 400  
 Ile Leu Thr Asn Pro Ile Ser Met Glu Lys Gln Ser His Met Val Ser  
 405 410 415  
 Leu Val Val Asn Val Pro Phe Gln Ile Gly Glu Lys Ala Leu Ile Ser

420

425

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# SUBSTITUTE SHEET



-59-

Arg Thr Val Lys Arg Ala Asn Glu Gly Glu Leu Lys Thr Gly Tyr Leu  
 785 790 795 800  
 Ser Ile Val Met Asp Pro Asp Glu Leu Pro Leu Asp Glu Arg Cys Glu  
 805 810 815  
 Arg Leu Pro Tyr Asp Ala Ser Lys Trp Glu Phe Pro Arg Asp Arg Leu  
 820 825 830  
 Lys Leu Gly Lys Pro Leu Gly Arg Gly Ala Phe Gly Gln Val Ile Glu  
 835 840 845  
 Ala Asp Ala Phe Gly Ile Asp Lys Thr Ala Thr Cys Lys Thr Val Ala  
 850 855 860  
 Val Lys Met Leu Lys Glu Gly Ala Thr His Ser Glu His Arg Ala Leu  
 865 870 875 880  
 Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly His His Leu Asn Val  
 885 890 895  
 Val Asn Leu Leu Gly Ala Cys Thr Lys Pro Gly Gly Pro Leu Met Val  
 900 905 910  
 Ile Val Glu Phe Cys Lys Phe Gly Asn Leu Ser Thr Tyr Leu Arg Gly  
 915 920 925  
 Lys Arg Asn Glu Phe Val Pro Tyr Lys Ser Lys Gly Ala Arg Phe Arg  
 930 935 940  
 Gln Gly Lys Asp Tyr Val Gly Glu Leu Ser Val Asp Leu Lys Arg Arg  
 945 950 955 960  
 Leu Asp Ser Ile Thr Ser Ser Gln Ser Ser Ala Ser Ser Gly Phe Val  
 965 970 975  
 Glu Glu Lys Ser Leu Ser Asp Val Glu Glu Glu Glu Ala Ser Glu Glu  
 980 985 990  
 Leu Tyr Lys Asp Phe Leu Thr Leu Glu His Leu Ile Cys Tyr Ser Phe  
 995 1000 1005  
 Gln Val Ala Lys Gly Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His  
 1010 1015 1020  
 Arg Asp Leu Ala Ala Arg Asn Ile Leu Leu Ser Glu Lys Asn Val Val  
 1025 1030 1035 1040  
 Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp  
 1045 1050 1055  
 Tyr Val Arg Lys Gly Asp Ala Arg Leu Pro Leu Lys Trp Met Ala Pro  
 1060 1065 1070  
 Glu Thr Ile Phe Asp Arg Val Tyr Thr Ile Gln Ser Asp Val Trp Ser  
 1075 1080 1085  
 Phe Gly Val Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr  
 1090 1095 1100  
 Pro Gly Val Lys Ile Asp Glu Glu Phe Cys Arg Arg Leu Lys Glu Gly  
 1105 1110 1115 1120  
 Thr Arg Met Arg Ala Pro Asp Tyr Thr Thr Pro Glu Met Tyr Gln Thr  
 1125 1130 1135  
 Met Leu Asp Cys Trp His Glu Asp Pro Asn Gln Arg Pro Ser Phe Ser

-60-

1140	1145	1150
Glu Leu Val Glu His Leu Gly	Asn Leu Leu Gln Ala Asn Ala Gln Gln	
1155	1160	1165
Asp Gly Lys Asp Tyr Ile Val Leu Pro Met Ser Glu Thr Leu Ser Met		
1170	1175	1180
Glu Glu Asp Ser Gly Leu Ser Leu Pro Thr Ser Pro Val Ser Cys Met		
1185	1190	1195
Glu Glu Glu Glu Val Cys Asp Pro Lys Phe His Tyr Asp Asn Thr Ala		
1205	1210	1215
Gly Ile Ser His Tyr Leu Gln Asn Ser Lys Arg Lys Ser Arg Pro Val		
1220	1225	1230
Ser Val Lys Thr Phe Glu Asp Ile Pro Leu Glu Glu Pro Glu Val Lys		
1235	1240	1245
Val Ile Pro Asp Asp Ser Gln Thr Asp Ser Gly Met Val Leu Ala Ser		
1250	1255	1260
<del>Glu Glu Leu Lys Thr Leu Glu Asp Arg Asn Lys Leu Ser Pro Ser Phe</del>		
<del>1265</del>	<del>1270</del>	<del>1275</del>
Gly Gly Met Met Pro Ser Lys Ser Arg Glu Ser Val Ala Ser Glu Gly		
1285	1290	1295
Ser Asn Gln Thr Ser Gly Tyr Gln Ser Gly Tyr His Ser Asp Asp Thr		
1300	1305	1310
Asp Thr Thr Val Tyr Ser Ser Asp Glu Ala Gly Leu Leu Lys Met Val		
1315	1320	1325
Asp Ala Ala Val His Ala Asp Ser Gly Thr Thr Leu Gln Leu Thr Ser		
1330	1335	1340
Cys Leu Asn Gly Ser Gly Pro Val Pro Ala Pro Pro Pro Thr Pro Gly		
1345	1350	1355
Asn His Glu Arg Gly Ala Ala		
1365		

-61-

WHAT IS CLAIMED IS:

1. A recombinant DNA vector containing a nucleotide sequence that encodes a Flk-1 operatively associated with a regulatory sequence that controls gene expression in a host.
2. A recombinant DNA vector containing a nucleotide sequence that encodes a Flk-1 fusion protein operatively associated with a regulatory sequence that controls gene expression in a host.
3. An engineered host cell that contains the recombinant DNA vector of Claims 1 or 2.
4. An engineered cell line that contains the recombinant DNA expression vector of Claim 1 and expresses Flk-1.
5. The engineered cell line of Claim 3 which expresses the Flk-1 on the surface of the cell.
6. An engineered cell line that contains the recombinant DNA expression vector of Claim 2 and expresses the Flk-1 fusion protein.
7. The engineered cell line of Claim 6 that expresses the Flk-1 fusion protein on the surface of the cell.
8. A method for producing recombinant Flk-1, comprising:
  - (a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 1 and which expresses the Flk-1; and

-62-

- (b) recovering the Flk-1 gene product from the cell culture.

9. A method for producing recombinant Flk-1 fusion  
5 protein, comprising:

- (a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 2 and which expresses the Flk-1 fusion protein; and  
10 (b) recovering the Flk-1 fusion protein from the cell culture.

10. An isolated recombinant Flk-1 receptor protein.

15 11. A fusion protein comprising Flk-1 linked to a heterologous protein or peptide sequence.

12. An oligonucleotide which encodes an antisense sequence complementary to a portion of the Flk-1  
20 nucleotide sequence, and which inhibits translation of the Flk-1 gene in a cell.

13. The oligonucleotide of Claim 12 which is complementary to a nucleotide sequence encoding the amino  
25 terminal region of the Flk-1.

14. A monoclonal antibody which immunospecifically binds to an epitope of the Flk-1.

30 15. The monoclonal antibody of Claim 14 which competitively inhibits the binding of VEGF to the Flk-1.

16. The monoclonal antibody of Claim 14 which is  
35 linked to a cytotoxic agent.

-63-

17. The monoclonal antibody of Claim 14 which is linked to a radioisotope.

18. A method for screening and identifying  
5 antagonists of VEGF, comprising:

(a) contacting a cell line that expresses Flk-1 with a test compound in the presence of VEGF; and

10 (b) determining whether the test compound inhibits the binding and cellular effects of VEGF on the cell line,

in which antagonists are identified as those compounds that inhibit both the binding and cellular effects of VEGF on the cell line.

15

19. A method for screening and identifying agonists of VEGF, comprising:

(a) contacting a cell line that expresses the Flk-1 with a test compound in the presence and in the absence of VEGF;

20

(b) determining whether, in the presence of VEGF, the test compound inhibits the binding of VEGF to the cell line; and

25

(c) determining whether, in the absence of the VEGF, the test compound mimics the cellular effects of VEGF on the cell line,

in which agonists are identified as those test compounds that inhibit the binding but mimic the cellular effects of VEGF on the cell line.

30

20. The method according to Claims 18 or 19 in which the cell line is a genetically engineered cell line.

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-64-

21. The method according to Claims 18 or 19 in which the cell line endogenously expresses the Flk-1.

22. A method for screening and identifying  
5 antagonists of VEGF comprising:

- (a) contacting Flk-1 protein with a random peptide library such that Flk-1 will recognize and bind to one or more peptide species within the library;
- 10 (b) isolating the Flk-1/peptide combination;
- (c) determining the sequence of the peptide isolated in step c; and
- (d) determining whether the test compound inhibits the binding and cellular effects  
15 of VEGF,

in which antagonists are identified as those peptides that inhibit both the binding and cellular effects of VEGF.

20 23. A method for screening and identifying agonists of VEGF comprising:

- (a) contacting Flk-1 protein with a random peptide library such that Flk-1 will recognize and bind to one or more peptide  
25 species within the library;
- (b) isolating the Flk-1/peptide combination;
- (c) determining the sequence of the peptide isolated in step c; and
- (d) determining whether, in the absence of the  
30 VRGF, the peptide mimics the cellular effects of VEGF,

in which agonists are identified as those peptides that inhibit the binding but mimic the cellular effects of Flk-1.

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-65-

24. The method according to Claims 22 or 23 in which the Flk-1 protein is genetically engineered.

25. A method of modulating the endogenous enzymatic activity of the tyrosine kinase Flk-1 receptor in a mammal comprising administering to the mammal an effective amount of a ligand to the Flk-1 receptor protein to modulate the enzymatic activity.

26. The method of Claim 25 in which the ligand to the Flk-1 receptor is VEGF.

27. The method of Claim 25 in which the ligand to the Flk-1 receptor is a VEGF agonist.

15

28. The method of Claim 25 in which the ligand to the Flk-1 receptor is an antagonist of VEGF.

29. The antagonist of Claim 28 that is a monoclonal antibody which immunospecifically binds to an epitope of Flk-1.

30. The antagonist of Claim 28 that is a soluble Flk-1 receptor.

25

31. The method of Claim 25 in which the enzymatic activity of the receptor protein is increased.

32. The method of Claim 25 in which the enzymatic activity of the receptor protein is decreased.

33. The method of Claim 31 in which the ligand stimulates endothelial cell proliferation.

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-66-

34. The method of Claim 32 in which the ligand inhibits endothelial cell proliferation.

35. The method of Claim 32 in which the ligand  
5 inhibits angiogenesis.

36. A recombinant vector containing a nucleotide sequence that encodes a truncated Flk-1 which has dominant-negative activity which inhibits the cellular  
10 effects of VEGF binding.

37. The recombinant vector of claim 36 containing a nucleotide sequence encoding amino acids 1 through 806 of Flk-1.

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38. The recombinant vector of claim 36 in which the vector is a retrovirus vector.

39. The recombinant vector of claim 38 containing  
20 a nucleotide sequence encoding amino acids 1 through 806 of Flk-1.

40. An engineered cell line that contains the recombinant DNA vector of Claim 36 and expresses  
25 truncated Flk-1.

41. An engineered cell line that contains the recombinant vector of Claim 38 or 39 and produces infectious retrovirus particles expressing truncated  
30 Flk-1.

42. An isolated recombinant truncated Flk-1 receptor protein which has dominant-negative activity which inhibits the cellular effects of VEGF binding.  
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-67-

43. A method of modulating the cellular effects of VEGF in a mammal comprising administrating to the mammal an effective amount of truncated Flk-1 receptor protein which inhibits the cellular effects of VEGF binding.

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1/26

FLK-1	866	ILIHGHHLNVNLLGACTKPGGPLMWIVEFSKFGNLSYLRGRNEFVPYKSGARFRQ
KDR		-----C-D-----S-----I-----
TKR-C		-----C-----S-----
FLK-1	926	GKDYVGEISVOLKRRLDSTITSSQSSASSGFVEEKSLSDVEEEEAEEEL YKDFLTLEHL IC
KDR		-----AIP-----P-D-----
TKR-C		-----
FLK-1	986	YSFQVAKGMEFLASRKC IHRDLAARNILLSEKNWK ICDFGLARD IYKOPDYVRKGDARL
KDR		-----
TKR-C		-----

FIG.1

2/26

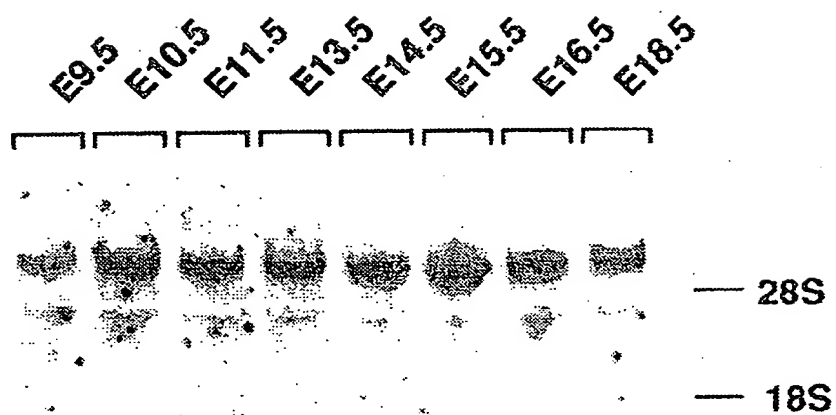


FIG. 2A

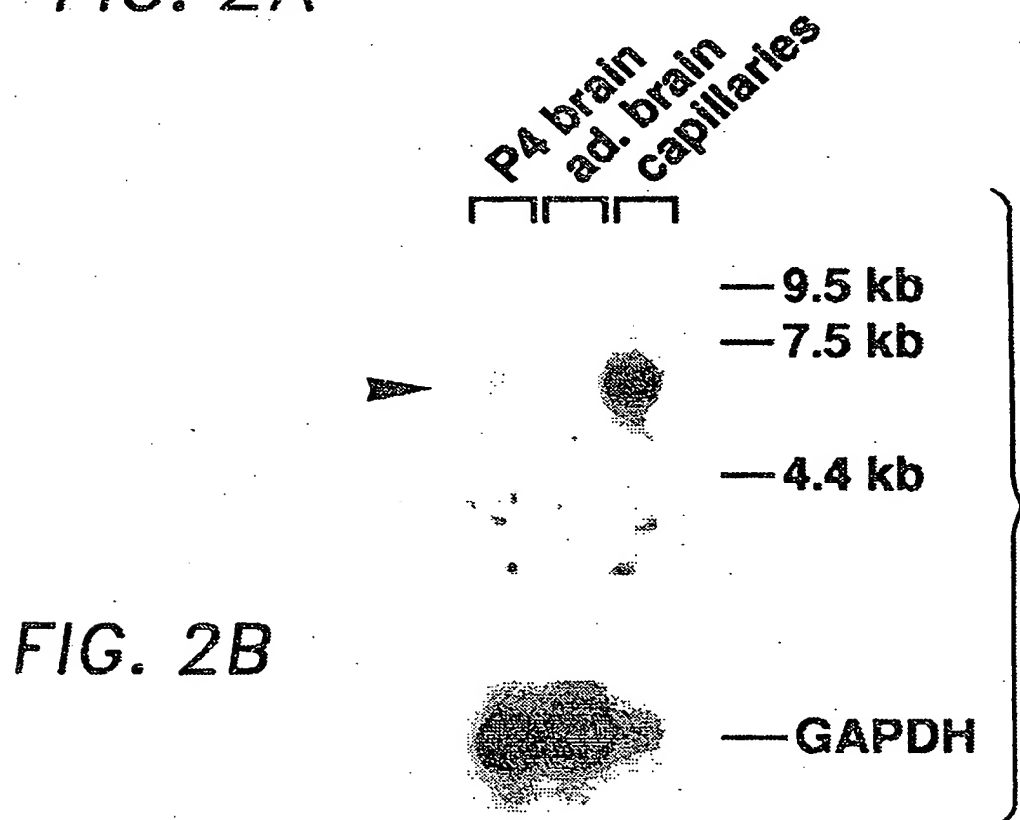


FIG. 2B

3/26

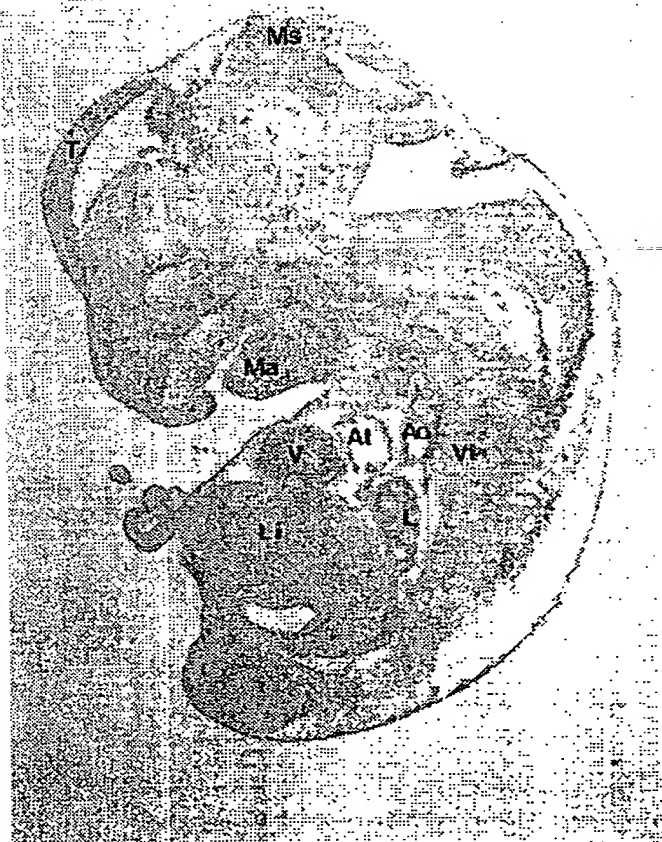


FIG. 3A

4/26

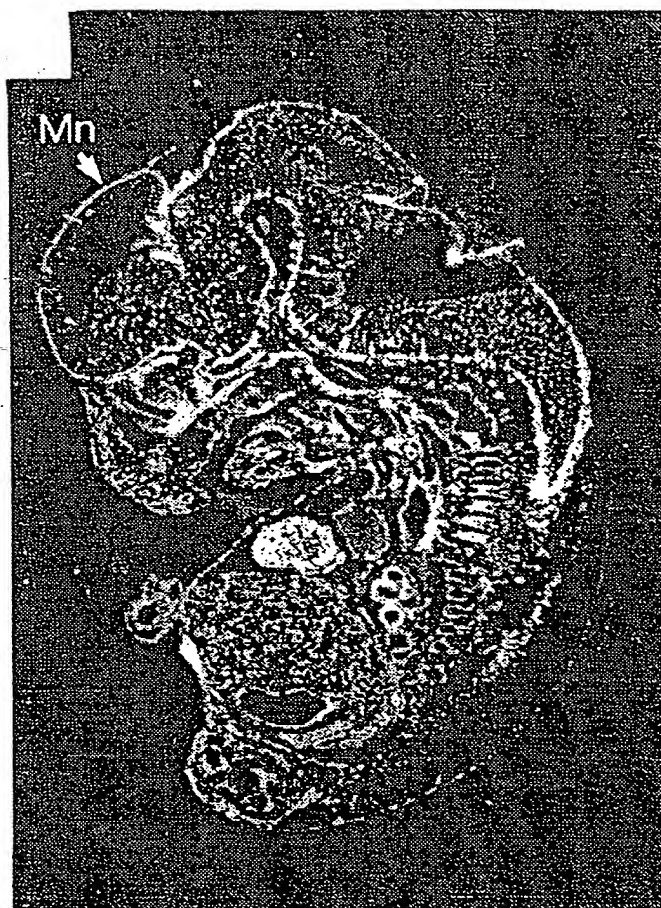


FIG. 3B

5/26



FIG. 3C

6/26

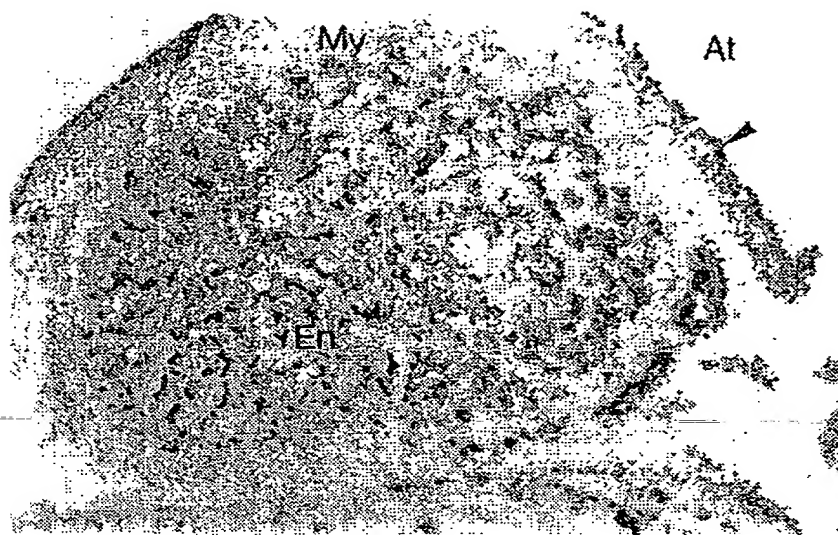


FIG. 4A



FIG. 4B

7/26



FIG. 4D



FIG. 4C



8/26

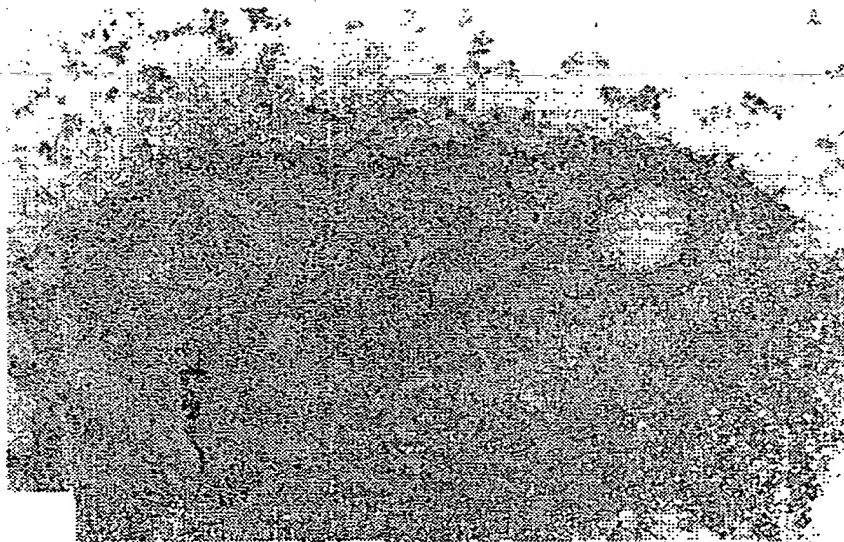


FIG. 4E

9/26

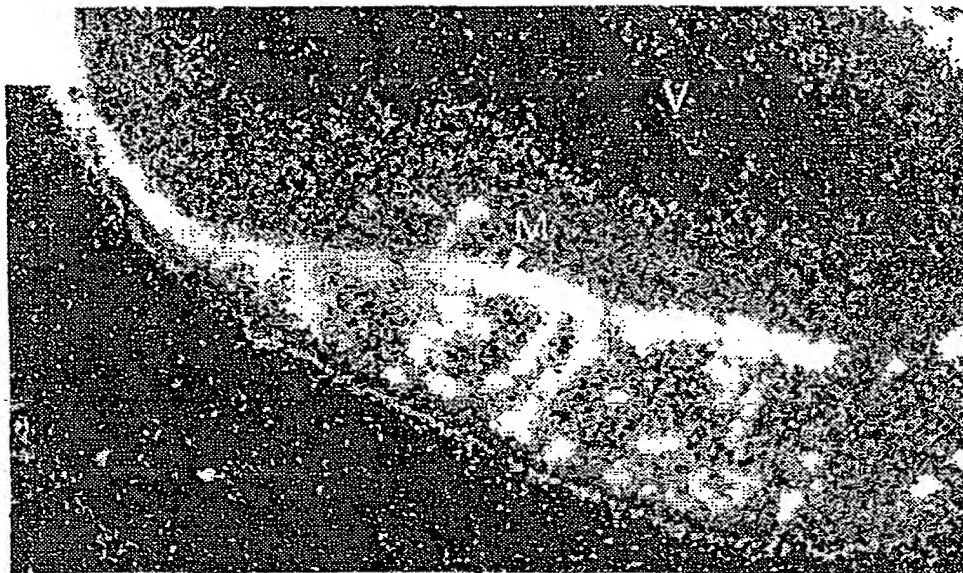


FIG. 5A

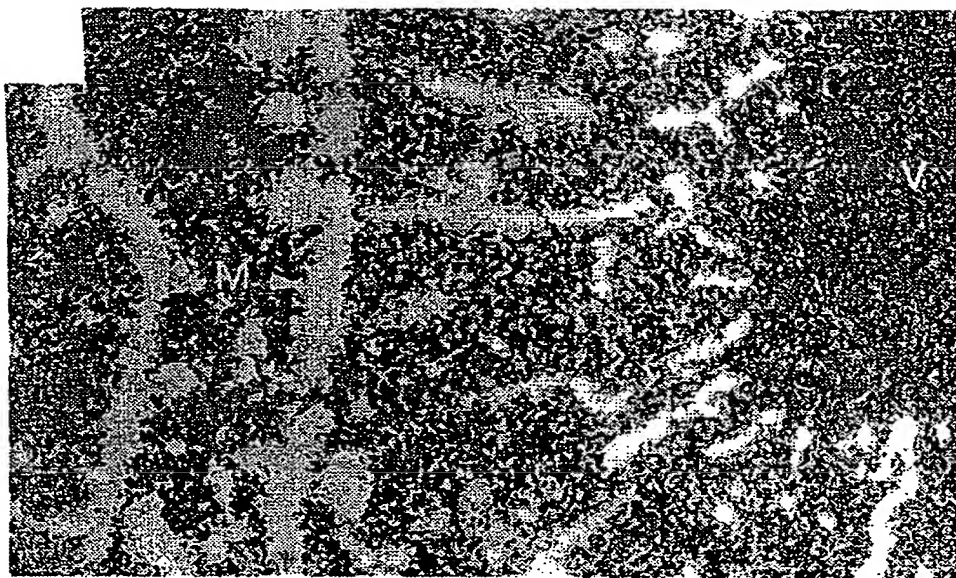
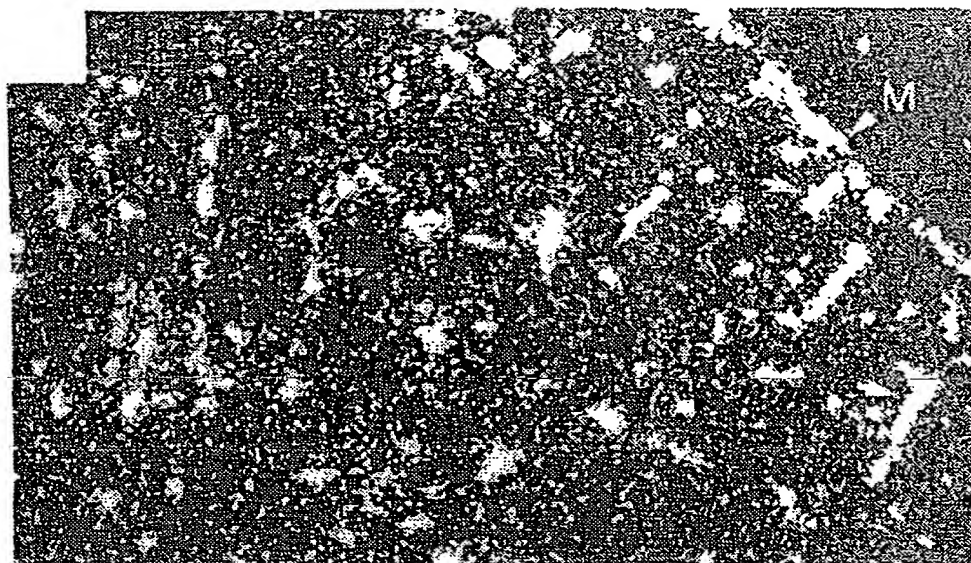
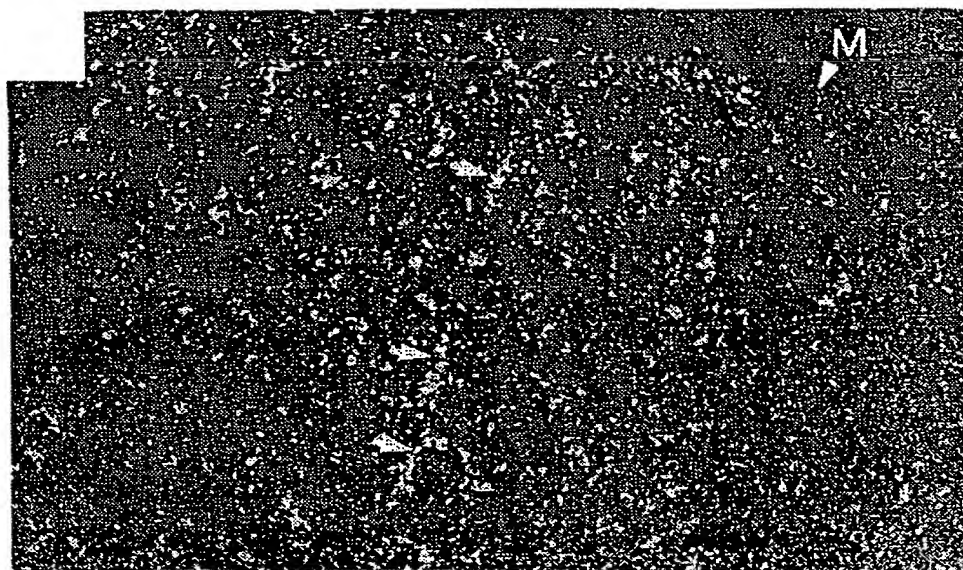


FIG. 5B

10/26



*FIG. 5C*



*FIG. 5D*

11/26

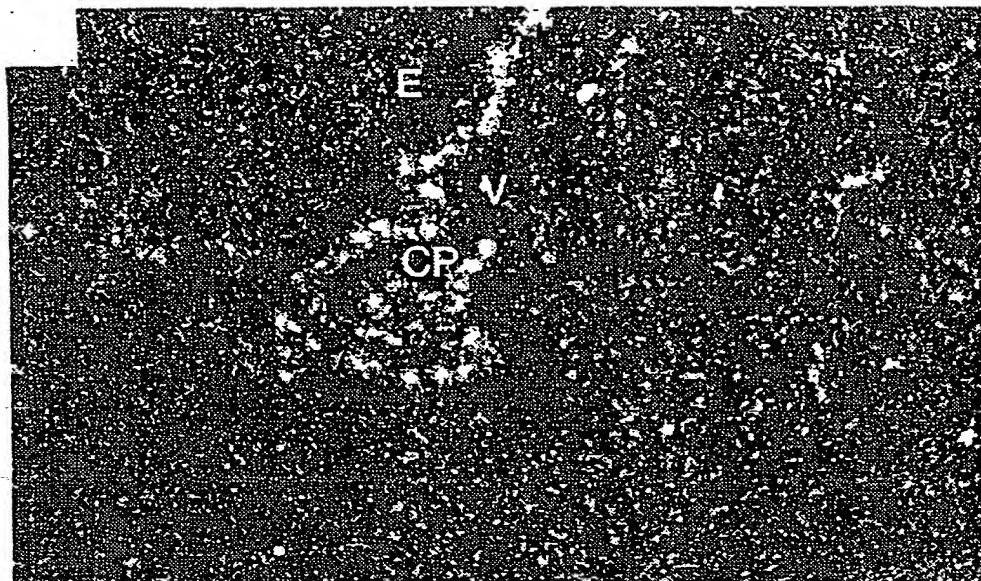


FIG. 6A

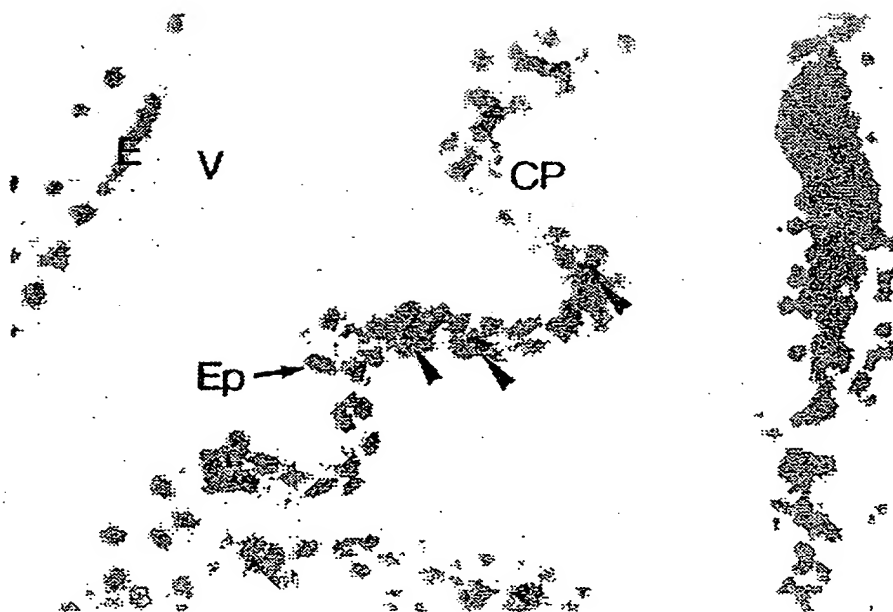


FIG. 6B

12 / 26

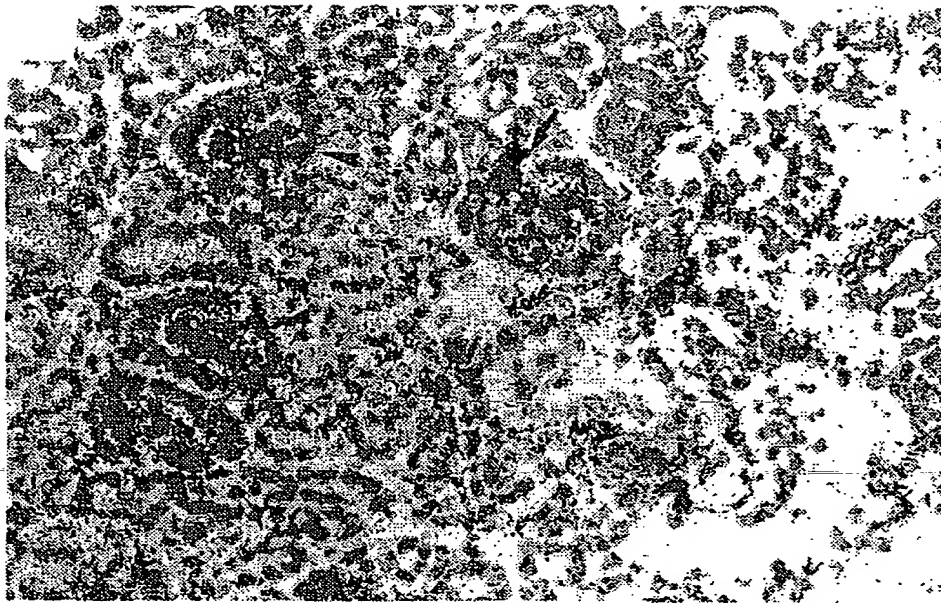


FIG. 7A

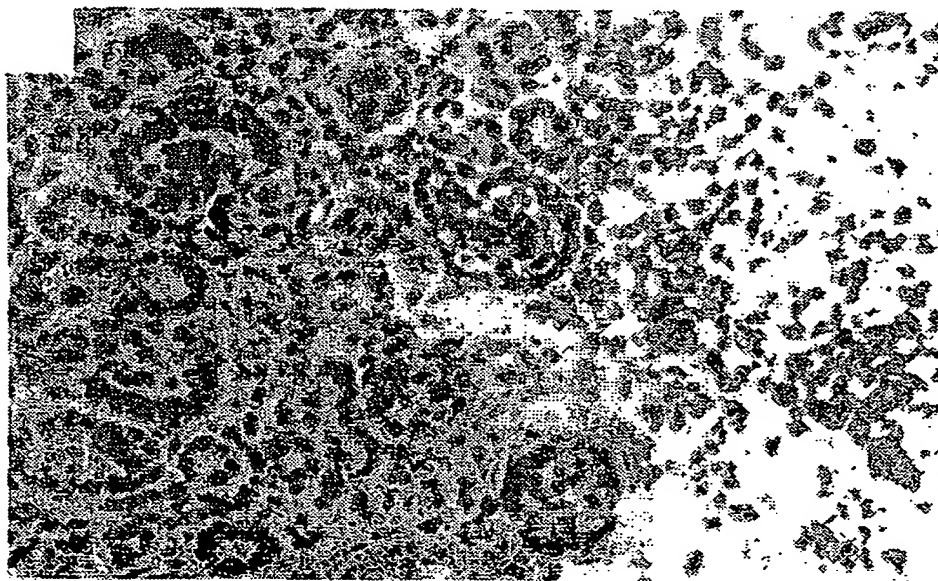


FIG. 7B

13/26

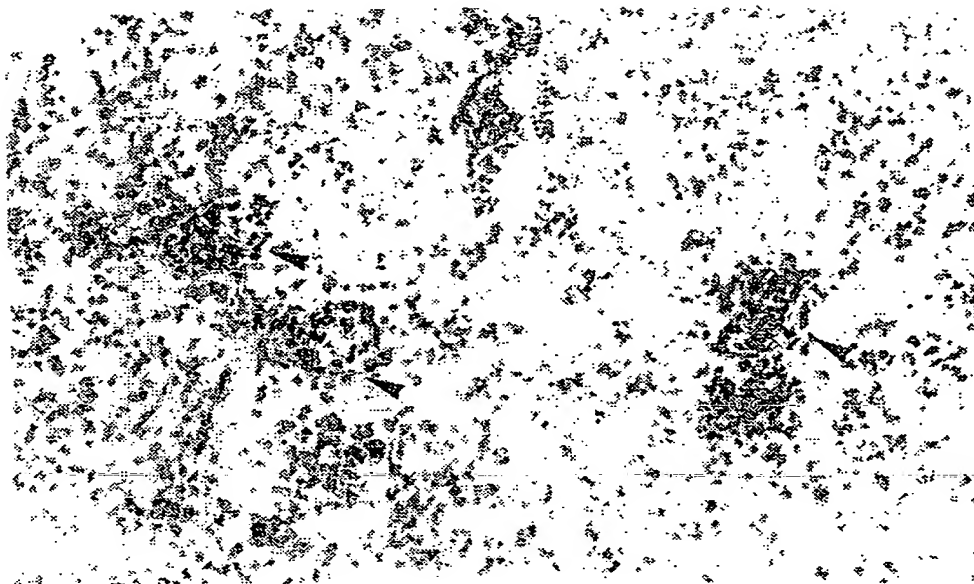


FIG. 7C

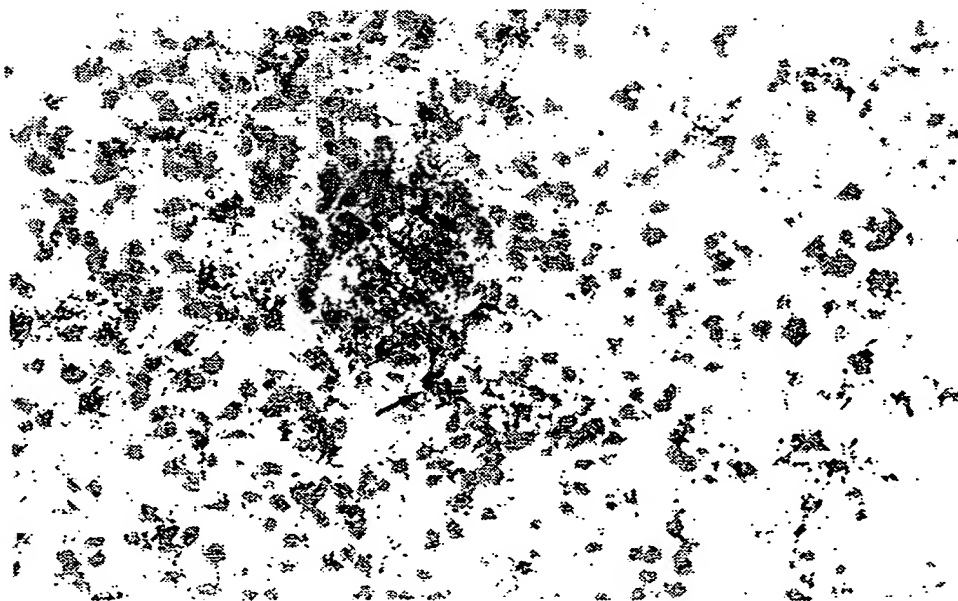


FIG. 7D



14/26

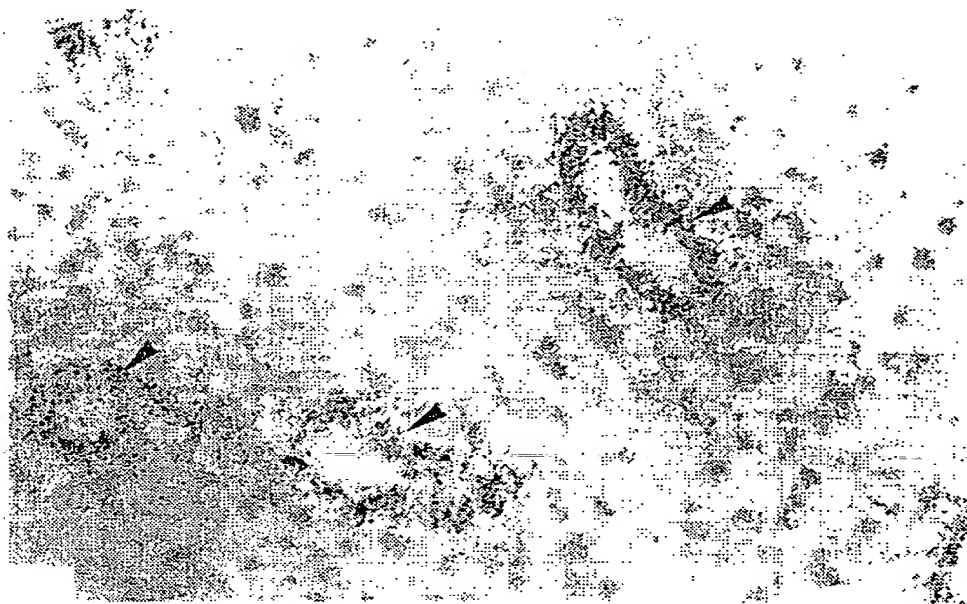


FIG. 8B

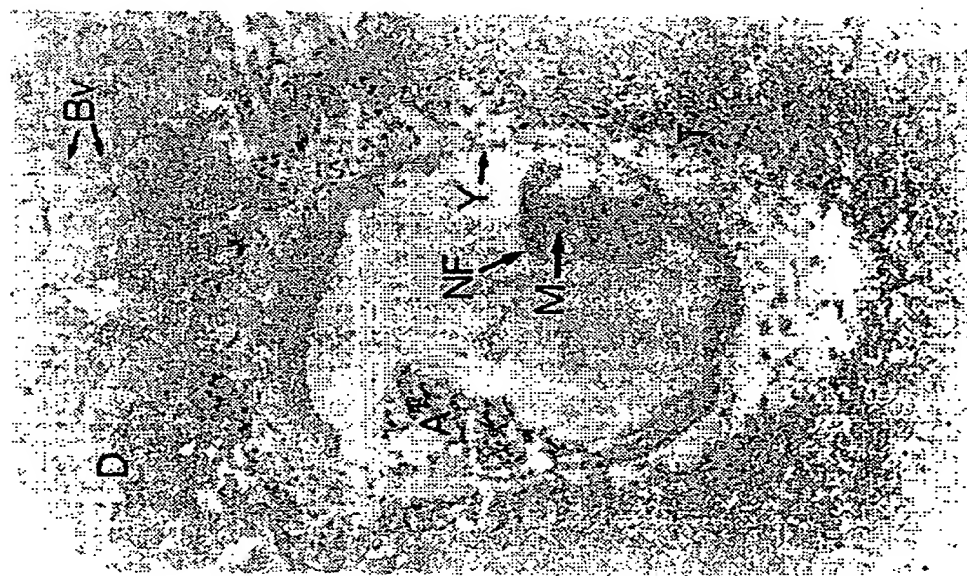


FIG. 8A

15/26

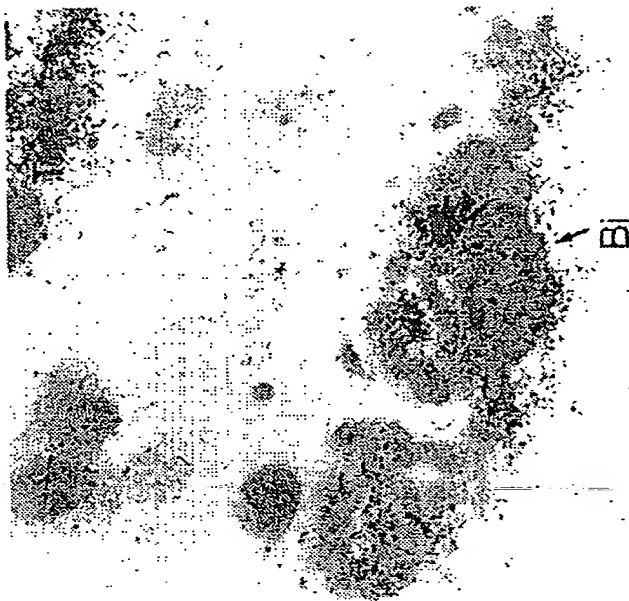


FIG. 8D

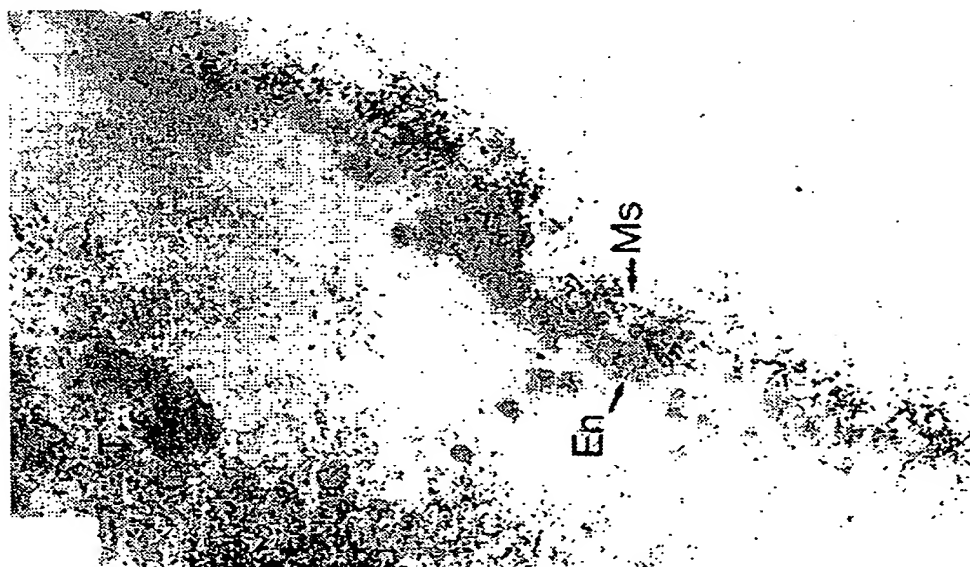
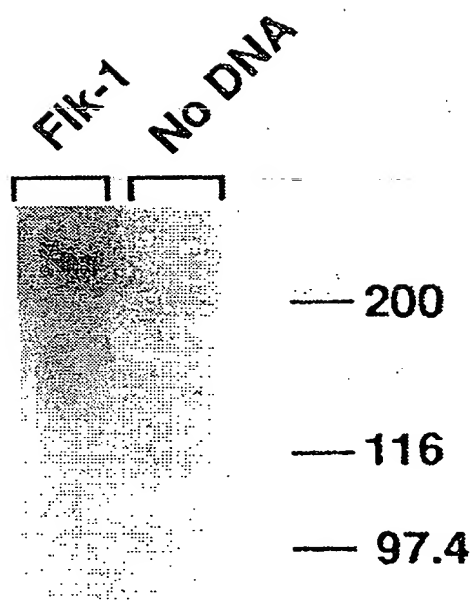


FIG. 8C



16/26



**FIG. 9A**

17/26

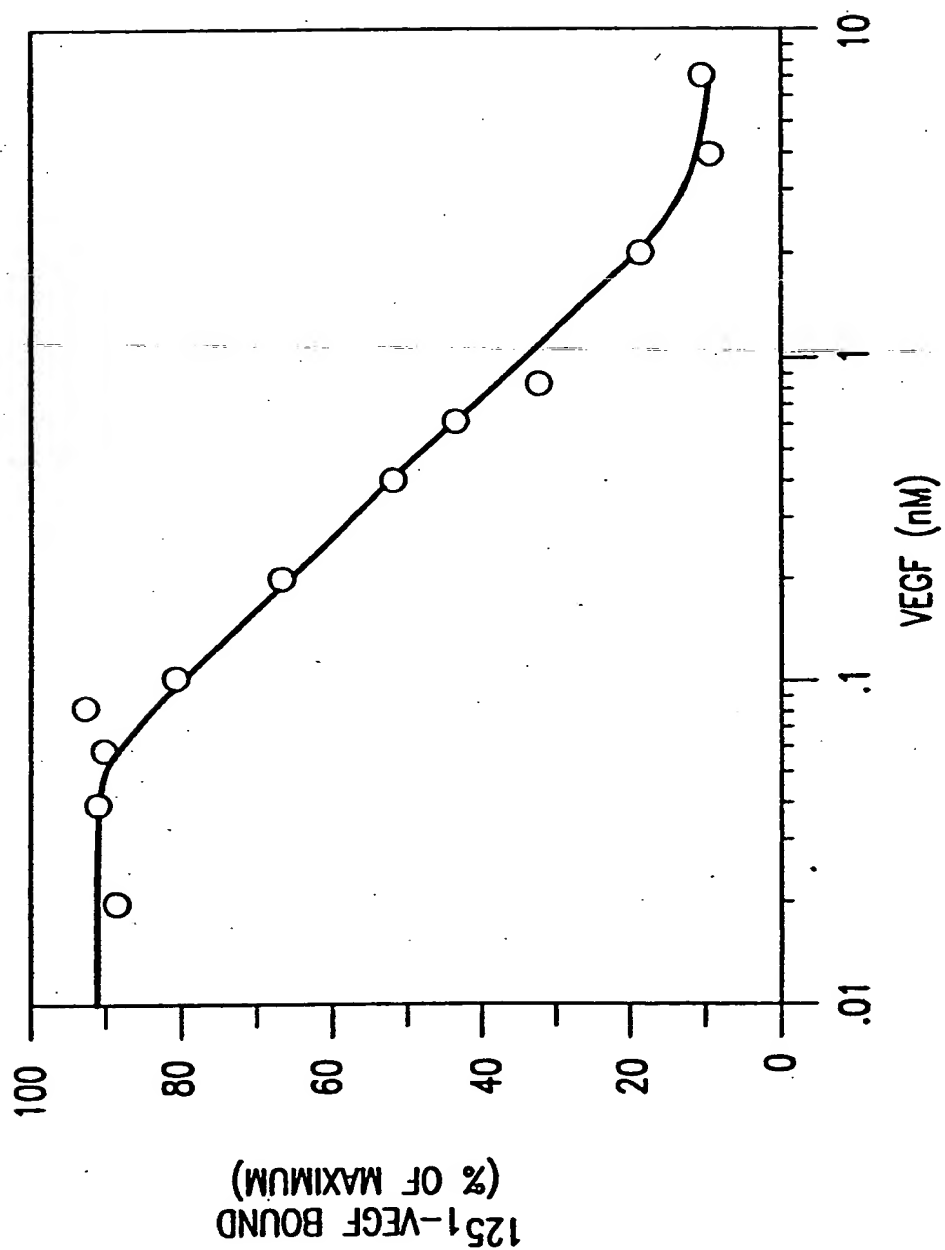
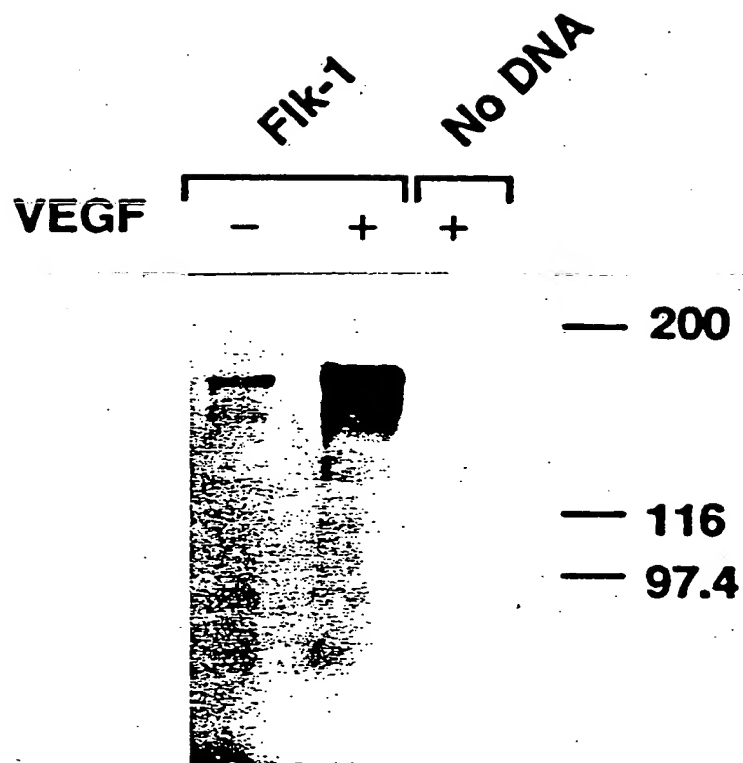


FIG.9B

18/26

**FIG. 10**

19/26

1 TATAGCGGAATTGGGTACGGGACCCCCCTCGAGGTGACGGTATCGATAAGCTTGATATCGAATTCGGGCCACACTGTGTCCCGCAGC 90  
 91 CCGGATAACCTGGCTGACCCGATTCCGGGACACGGCTGACAGCCGGCGCTGGAGCCAGGGCGCGGTGCCCCGGCTCTCCCGGTCTT 160  
 181 GCGCTGCGGGGCCATACCGCTCTGTGACTTCTTTGCGGGCAGCGACGAGAAGGAGTCTGTGCCTGAGAACTGGGCTCTGTGCCCCA 270  
 M E S K A L L A V A L W F C V H T R A A S V G L T 25  
 271 CCGCGCAGGTGCAGATGAGAGCAAGCGCTGCTAGCTGTGGCTCTGTGGTTCTGCGTGGAGACCCGAGCGGCTCTGTGGTTTACT 360  
 26 G D F L H P P K L S T Q K D I L T I L A N T T L Q I T C R G 55  
 361 CCGGATTTTCTCCATCCCCCAAGCTCAGCACACAGAAGACATACTGACAATTTGGCAAATAACAACCTTCAGATTACTTGCAGGGGA 450  
 56 Q R D L D K L M P N A Q R D S E H R V L V T E C G G G D S I 85  
 451 CAGCGGACCTGGACTGGCTTTGCGCCAATGCTCAGGTGATTCTGAGGAAAGGCTATTGGTGAATGCGGGCGTGGTACAGTATC 540  
 86 F C K T L T I P R V V G N D T G A Y K C S Y R D V D I A S T 115  
 541 TTCTGCAAAACACTCACCATTCCCAGGTGGTTGCAATGATACTGAGCGCTACAAGTGCTGCTACCGGACGTGACATAGCCTCCACT 630  
 116 V Y V Y V R D Y R S P F I A S V S D Q H G I V Y I T E N K N 145  
 631 GTTATGTCTATGTTGAGATTACAGATCACCATTATGCGCTCTGTGCTAGTACCAGCATGGCATGCTGTACATACCGAGAACAAAGAAC 720  
 146 K T V V I P C H G S I S N L N V S L C A R Y P E K R F V P D 175  
 721 AAACTGTGGTGATCCCCGCGAGGTGGATTTCAAACCTCAATGTGTCTCTTTGCGCTAGGTATCCAGAAAAGAGATTGTTCCGGAT 810  
 176 G N R I S K D S H I G F T L P S Y M I S Y A G M V F C E A K 205  
 811 GGAAACAGAAATTCCTGGGACAGGAGATAGCCTTTACTCTCCCGAGTTACATGATCAGCTATGCGCGCATGGTCTTCTGTGAGGCAAG 900  
 206 I N D K T Y Q S I M Y I V V V V G Y R I Y D V I L S P P H H 235  
 901 ATCAATGATGAACCTATCAGTCTATCATGTACATAGTGTGGTGTAGGATATAGGATTTATGATGTGATTCTGAGCCCCCGCATGAA 990  
 236 I K L S A G K K L V L N C T A R T E L N V G L D F T M H S P 265  
 991 ATTGAGCTATCTGCGGAGAAAACTTGTCTTAAATTGTACAGCGAGAACAGAGCTCAATGTGGCGCTTGATTTCACCTGGCACTCTCCA 1080  
 266 P S K S H H K K I V N R D V K P F P G T V A K M F L S T L T 295  
 1081 CCTTCAAGTCTCATATAAGAGATTGTAACCGGGATGTGAACCCCTTCTCGGACTGTGGGGAAGATGTTTTGAGCACCTTGACA 1170  
 296 I E S V T K S D Q G E Y T C V A S S G R M I K R N R T F V R 325  
 1171 ATAGAAGTGTACCAAGAGTGACCAAGCGGAATACACCTGTGTAGCGTCCAGTGGACCGATGATCAAGAGAAATAGAACATTTGTCCCA 1260

FIG.11A

20/26

326 V H T K P F I A F G S G M K S L V E A T V G S Q V R I P V K 355  
 1261 GTTCACACAAACCCCTTTTATTGCTTTCCGTAGTGGGATGAAATCTITGGTGAAGCCACAGTGGGCAGTCAAGTCCGAATCCCTGTGAAG 1350  
 356 Y L S Y P A P D I K N Y R N G R P I E S N Y T M I V G D K L 385  
 1351 TATCTCAGTTACCCAGCTCCTGATATCAAATCGTACAGAAATGGAAGGCCATTGAGTCCAACACACAATGATTGTTGCCGATGAAGTC 1440  
 386 T I M K V T K R D A Q N Y T V I L T N P I S N E K Q S H M V 415  
 1441 ACCATCATCGAAGTCACTGAAAGAGATGCAGGAACTACCGGTCATCCTACCAACCCCATTTCAATGGAGAAACAGGCCACATGGTC 1530  
 416 S L V V K V P P Q I G E K A L I S P M D S Y Q Y G T M Q Y L 445  
 1531 TCTCTGGTTGTGAATGTCCACCCAGATCGGTGAGAAAGCCTTGATCTCCCTATGGATTCTTACCAGTATGGGACCATGCAGACATIG 1620  
 446 T C T V Y A N P P L H H I Q N Y N Q L E E A C S Y R P G Q T 475  
 1621 ACATGCACAGTCTAAGCCCAACCCCTCCCTGCACCACATCCAGTGGTACTGGCAGCTAGAAGAAGCCTGCTCCTACAGACCCGGCCAAACA 1710  
 476 S P Y A C K E K R H V E D F Q G G N K I E V T K N Q Y A L I 505  
 1711 AGCCCGTATGCTTGTAAAGAATGGAGACACCGTGGAGGATTTCCAGGGGGGAAACAAGATCGAAGTCACCAAAAACCAATATGCCCTGATT 1800  
 506 K G K N K T V S T L V I Q A A N V S A L Y K C E A I N K A G 535  
 1801 GAAGGAAAAACAAAACCTGAAGTACGCTGGTCATCCAAGCTGCCAAGCTGTCAGCGTTGTACAAATGTGAGCCATCAACAAAGCGGA 1890  
 536 R G E R V I S F H V I R G P E I T V Q P A A Q P T E Q E S V 565  
 1891 CGAGGAGACAGGGTCATCTCCTTCCATGTGATCAGGGTCCGAAATTACTGTGCAACCTGCTGCCAGCCAACTGAGCAGGAGAGTGTG 1980  
 566 S L L C T A D R N T F E N L T N Y K L G S Q A T S V H N G E 595  
 1981 TCCCTGTGTGCACTGCAGACAGAAATACGTTTGAGAACCTCAGTGGTACAGCTTGGCTCAGGGCAACATCGTCCACATGGGCGAA 2070  
 596 S L T P V C K N L D A L N K L M G T M F S N S T N D I L I V 625  
 2071 TCACTCACACCAGTTTGAAGAAGTTGGATGCTCTTTGAAACTGAATGGCACCATGTTTCTAACAGCACAAATGACATCTTGATTGTG 2160  
 626 A F Q N A S L Q D Q G D Y V C S A Q D K K T K K R H C L V K 655  
 2161 GCATTTCAGAATGCCCTCTCTGCAGGACCAAGCGACTATGTTGCTCTGCTCAAGATAAGAAGACCAAGAAAAGACATTGCCCTGGTCAA 2250  
 656 Q L I I L K R M A P H I T G N L S N Q T T T I Q E T I H V T 685  
 2251 CAGCTCATCATCTAGAGGGCATGGCACCATGATCACCGGAACTCGGAGAATCAGACAACAACCATTGGCGAGACCATTGAAGTCACT 2340  
 686 C P A S C N P T P N I T K F K D N E T L V E D S G I V L R D 715  
 2341 TGCCAGCATCTGGAATCCTACCCACACATTACATCGTTCAAAGACAACGAGACCCCTGGTAGAAGATTCAAGCATTTACTGAGAGAT 2430  
 716 G N R N L T I R R V R K E D G G L Y T C Q A C N V L G C A R 745  
 2431 CGGAACCCGAACCTGACTATCCGACGGTCAGGAAGGAGATGGAGCCCTCTACACCTGCCAGCCCTGCAATGTCTTGGCTGTGAAGA 2520

FIG.11B

21/26

746 A E T L F I I E G A Q H K T N L E V I I L V G T A V I A M F 775  
2521 GCGAGAGCGCTCTTCATAATAGAAGGTCCCGAGGAAAGACCAACTTGGAGTCATTATCCTCGTCCGCACTGCCAGTGATGCCATGCTC 2610

776 F M L L L V I V L R T V K R A N H G K L K T G Y L S I V M D 805  
2611 TTCTGGCTCCTTCTTGTTCATGTGCTACGGACCGTTAAGCGCGCAATGAAGCGGAAGTGAAGACAGCGCTACTTGTCTATTGTTCATGGAT 2700

806 F D K L P L D H R C K E L P Y D A S K N E F P R D R L K L G 835  
2701 CCAGATCAATTGCCCTTGGATGAGCGCTGTGAAGCGTTGCCCTATGATGCCAGCAAGTGGGAATTCGCCAGCGACCGCTGAAACTAGGA 2790

836 K F L G R G A F G Q V I E A D A F G I D K T A T C K T V A V 865  
2791 AAACCTCTTGGCGCGCGTGCCTTCGGCCAAGTGATTGAGGCAGACCGCTTTTGGAAATTGACAAGACAGCGACTTGCAAAACAGTAGCCGTC 2880

866 K M L K E G A T H S E H R A L M S K L K I L I H I G H H L M 895  
2881 AAGATGTTGAAAGGAGGAGCAACACACAGCGAGCATGAGCCCTCATGTCTGAAGTCAAGATCCTCATCCACATGGTCACCATCTCAAT 2970

896 V V N L L G A C T K P G G P L M V I V E F C K F G N L S T Y 925  
2971 GTGGTGAACCTCCTAGCGCGCTGCACCAAGCGCGAGCGCTCTCATGGTGATTGTGGAATTCGCAAGTTGCAAACTATCAACTTAC 3060

926 L E G K R N E F V P Y K S K G A R F R Q G K D Y V G K L S V 955  
3061 TTACCGCGCAAGAGAAATGAATTTGTTCCTATAAGAGCAAAGCGGACCGCTTCGCCAGCGCAAGCACTACGTTGGGAGCTCTCCGTG 3150

956 D L K R R L D S I T S S Q S S A S S G F V K H K S L S D V E 985  
3151 GATCTGAAAGACCGCTTGGACAGCATCACCAGCAGCGAGCTCTGCCAGCTCAGCGCTTTGTTCAGGAGAAATCCCTCAGTGATGTAGAG 3240

986 K K K A S K K L Y K D F L T L K H L I C Y S F Q V A K G M E 1015  
3241 GAAGAAGAAGCTTCTGAAGAACTGTACAAGCACTTCCTGACCTTGGAGCATCTCATCTGTACAGCTTCCAAGTGGCTAAGCGCATGGAG 3330

1016 F L A S R K C I H R D L A A R N I L L S E K N V V K I C D F 1045  
3331 TTCTTGGCATCAAGGAAGTGATCCACAGCGACCTGCCAGCAGGAACATTCTCCTATCGGAGAAGAATGTGGTTAAGATCTGTGACTTC 3420

1046 G L A R D I Y K D P D Y V R K G D A R L P L K K M A P E T I 1075  
3421 CGCTTGGCGCGGACATTTATAAGACCGGATTATGTCAGAAAAGAGATGCGCGACTCCCTTTGAAGTGGATGCGCGCGGAACCAT 3510

1076 F D R V Y T I Q S D V N S F G V L L N E I F S L G A S P Y P 1105  
3511 TTTCACAGATATACACAATTCAGAGCGATGTGCTCTTTCCGTGTGTGCTCTGGGAATATTTTCCCTACGTGCTCCCATACCT 3600

1106 G V K I D E E F C R R L K E G T R M R A P D Y T T P E M Y Q 1135  
3601 CGCGTCAAGATTGATGAAGAATTTGTAGGAGATTGAAGAAGCAACTAGAATGCGCGCTCTGACTACACTACCCAGAAATGTACCAG 3690

FIG.11C

SUBSTITUTE SHEET

1136 T M L D C N H E D P N Q R P S F S E L V E H L G N L L Q A N 1165  
3691 ACCATGCTGGACTGCTGGCATGAGGACCCCAACACAGACCCCTGTTTTCAGAGTTGGTGGAGCATTGGGAAACCTCCTGCAAGCAAAT 3780

1166 A Q Q D G K D Y I V L P M S E T L S M K E D S G L S L P T S 1195  
3781 GCGCAGCAGGATCCCAAGACTATATTGTTCTTCCAATGTCAGAGACACTGAGCATGGAAGAGCATTCTGGACTCTCCCTGCCCTACCTCA 3870

1196 P V S C M E E E H V C D P K Y H Y D N T A G I S H Y L Q N S 1225  
3871 CCTGTTCTGTATGAGGAAGAGAGTGTCGACCCCAAATTCATTATGACAACACAGCAGGAATCAGTCATTATCTCCAGAACAGT 3960

1226 K R K S R P V S V K T F H D I P L E E P E V K V I P D D S Q 1255  
3961 AAGCGAAGAGCCGCCAGTGAGTGTAACCAATTTGAAGATATCCATTGGAGGAACAGAGTAAAGTGATCCAGATGACAGCCAG 4050

1256 T D S G M V L A S E E L K T L E D R N K L S P S F G G M M P 1285  
4051 ACAGACAGTGGATGCTCTTGCATCAGAAGAGCTCAAACTCTGGAAGACAGGAACAATTATCTCATCTTTGGTGAATGATGCCC 4140

1286 S J S R E S V A S E G S B Q T S G T Q S G T G S D D T D T T 1315  
4141 AGTAAAGCAGGAGTCTGTGCCCTCGGAAGCTCCAACAGACAGTGGCTACCAGTCTGGTATCACTCAGATGACACAGACACCACC 4230

1316 V Y S S D E A G L L K M V D A A V H A D S G T T L Q L T S C 1345  
4231 GTGACTCCAGCAGCAGGACGACTTTTAAAGATGGTGGATGCTGCAGTTACGCTGACTCAGGACCACACTGCAGCTCACCTCCTGT 4320

1346 L N G S G P V P A P P P T P G N H E R G A A \* 1367  
4321 TTAATGGAAGTGTCTGTCCCGCTCCGCCCCCAACTCTGGAATCAGGAGAGGTGCTGTAGATTTTCAAGTGTCTTCTTTC 4410

4411 CACCACCCGAAGTAGCCACATTTGATTTTCATTTTGGAGGAGGACCTCAGACTGCAAGGAGCTTGTCTCAGGGCATTTCAGAGAA 4500

4501 GATGCCCATGACCAAGAATGTGTGACTCTACTCTCTTTTCCATTCAITTTAAAGTCCATATAATGTGCCCTGCTGTGCTCTACTAC 4590

4591 CAGTTAAAGCAAAGACTTTCAAACAGTGGACTCTGTCTCCAAGAAGTGGCAAGGCACCTCTGTGAACTGGATCGAATGGCAATG 4680

4681 CTTTGTGTGTGAGGATGGTGAGATGTCCAGGGCGAGTCTGTCTACCTTGGAGCCTTTGTGAGGATGGGGCTATGAGCCAAGTGT 4770

4771 TAAGTGTGGATGTGACTGGGAGGAAGCAAGCGCAAGTGGCTGGAGAGCGGTGGAGCCTGCAGATGCATTGTCTGGCTCTGGTGG 4860

4861 AGGTGGCTTGTGGCTGTGAGAAAGCAAGCGGGCGGAGGTTTGGTTTGGAAAGTTGGCTGTCTTCAGATCGGTTACAG 4950

4951 GCGAGTCCCTGTGGGTTTCTACTCTAATGAGAGTTCTTCCGAGCTTACCTGTCTCTGGCTGGCCCCAGGAAGGAATGATG 5040

5041 CAGCTTGCTCCTTCTCATCTCTCAGGCTGTGCTTAATTCAGAACCAAAAAGAGAGGAAGCTGGCAGAGGCTCCTGACGGGGCGAA 5130

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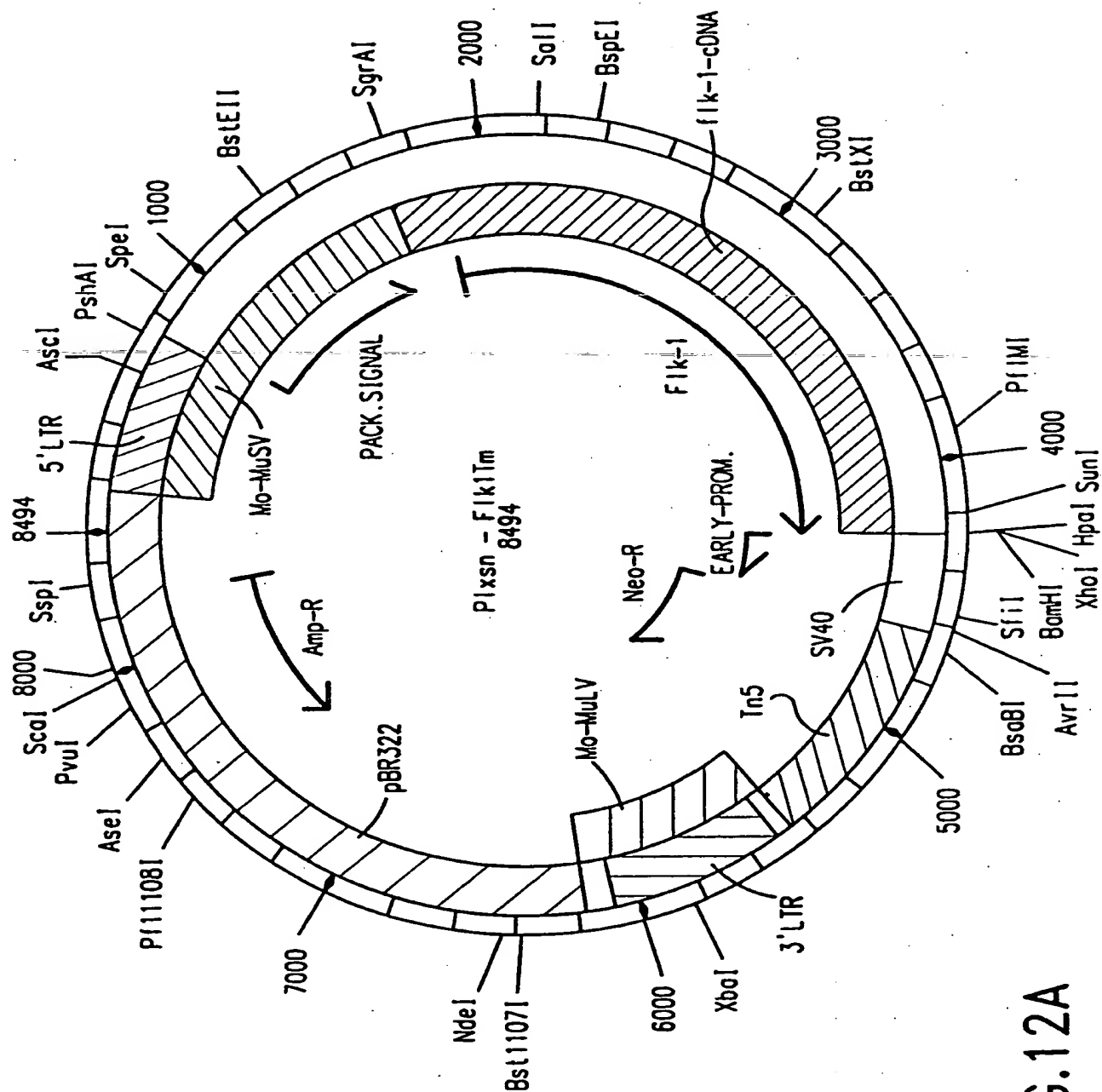
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5311 GATGGCAGAAAATCTTAATGGTTGGTTTGGCTCTCCAGATAATCACTAGCCAGATTTCGAAATTACTTTTTAGCCAGGTTATGATAAC 5400

5401 ATCTACTGTATCCTTTAGAATTTAACCTATAAACTATGTCTACTGTTTCTGCCCTGTGCTTATGTT 5470

FIG.11D

23 / 26



**FIG. 12A**



24/26

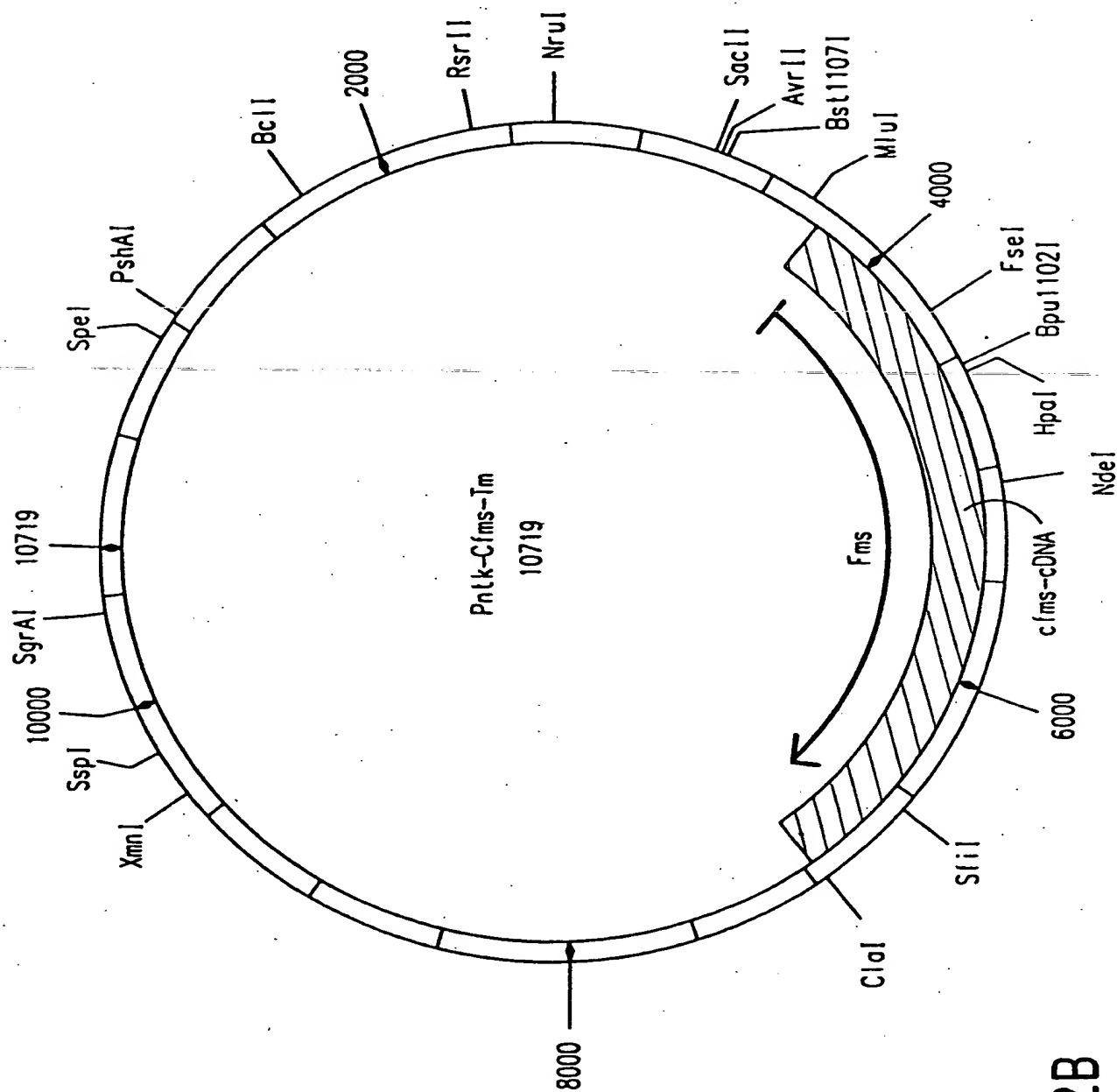


FIG.12B

25/26

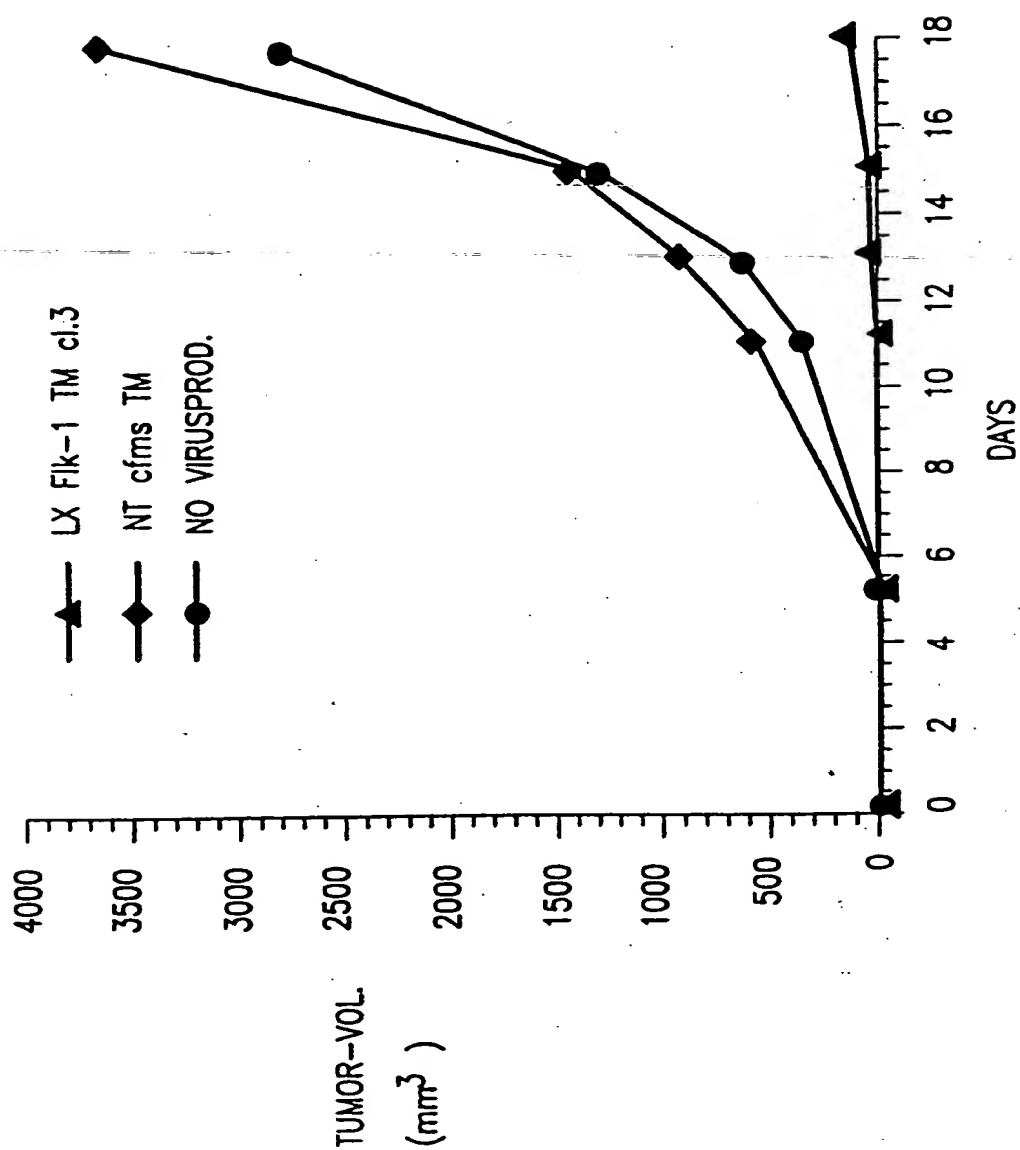


FIG.13

26/26

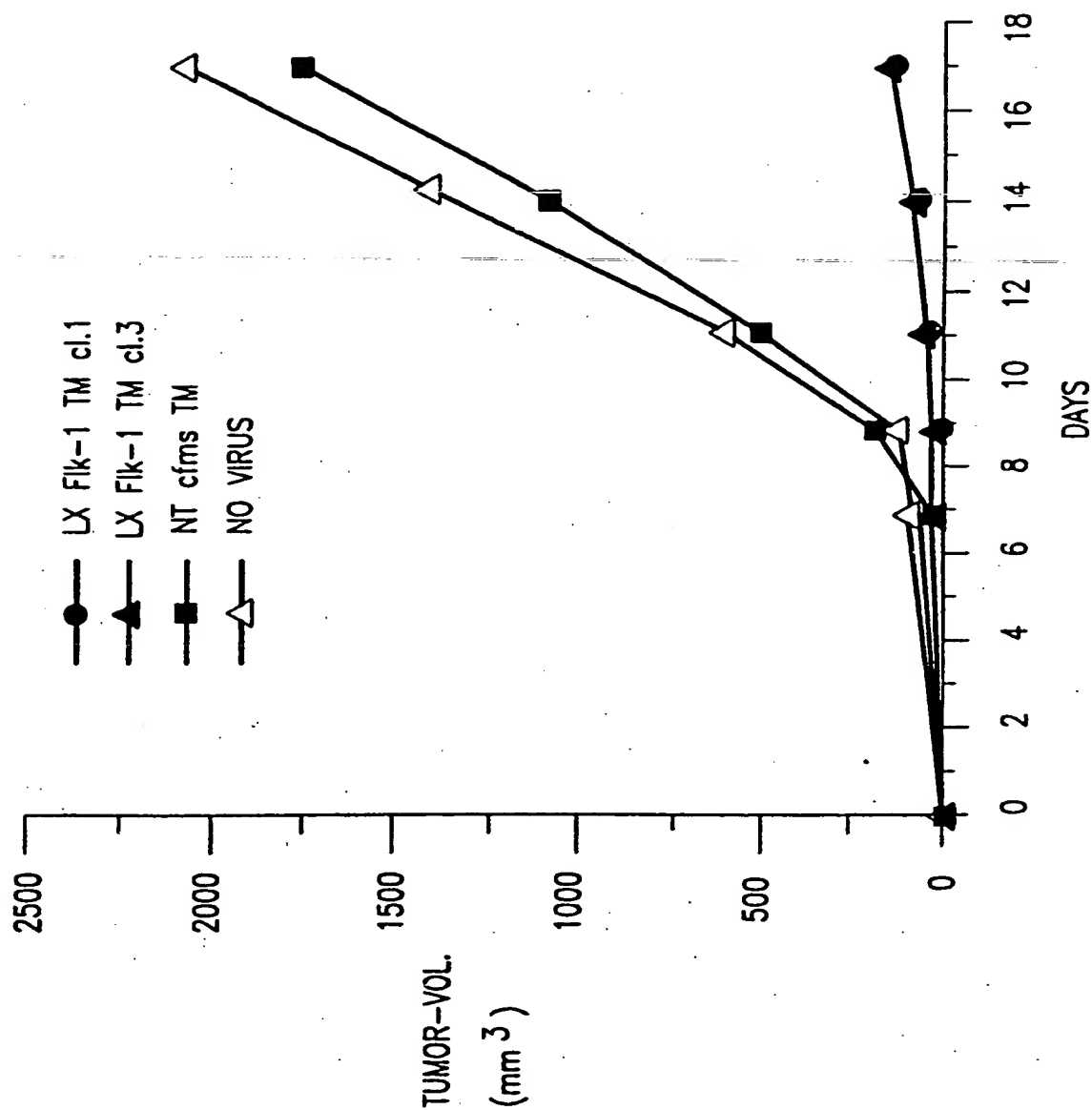


FIG.14

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 93/03191

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 C12N15/12 C07K13/00 C12P21/08 C12N15/86 C12Q1/68  
G01N33/567 A61K37/02 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 5 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, October 1991, WASHINGTON US pages 9026 - 9030 MATTHEWS, W. ET AL.; 'A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit.' see the whole document ---	1-11
X	WO,A,92 17486 (TRUSTEES OF PRINCETON UNIVERSITY, US) 15 October 1992 see the whole document ---	1-11, 14-37 12,13, 38,39,41
Y	---	
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

11 March 1994

Date of mailing of the international search report

15-04-1994

Name and mailing address of the ISA

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Authorized officer

Nauche, S

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/03191

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 03459 (SLOAN KETTERING INSTITUTE OF CANCER, US) 5 March 1992 see the whole document ---	12,13
Y	BIOTECHNOLOGY vol. 3, no. 8 , August 1985 , NEW YORK US pages 689 - 693 MC CORMICK, D.; 'Human gene therapy : the first round' see the whole document ---	38,39,41
P,X	CELL vol. 72 , 26 March 1993 , CAMBRIDGE, MA US pages 835 - 846 MILLAUER, B., WIZIGMANN-VOOS, S., SCHNURCH, H., MARTINEZ, R., MOLLER, N.P., RISAU, W., AND ULLRICH, A.; 'High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis.' see the whole document -----	1-43

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 93/03191

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 25-28, 31-35, 43 are directed to a method of treatment of the human/animal body as well as diagnostic methods (Rule 39.1 (iv) PCT) the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 93/03191

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9217486	15-10-92	US-A- 5185438	09-02-93
		AU-A- 1924892	02-11-92
		CA-A- 2107463	03-10-92
		EP-A- 0580760	02-02-94
		WO-A- 9300349	07-01-93
		US-A- 5283354	01-02-94
		US-A- 5270458	14-12-93
		AU-A- 2296292	25-01-93
		AU-A- 3139493	15-06-93
		WO-A- 9310136	27-05-93
WO-A-9203459	05-03-92	AU-A- 8510691	17-03-92
		CA-A- 2090469	28-02-92
		EP-A- 0546054	16-06-93